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Walia

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(54) **MULTIPLEX ASSAYS WITH MULTIPLE LUCIFERASES REPORTERS AND USES THEREOF**

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C12Q 1/66 (2006.01)
C12N 9/02 (2006.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/66*** (2013.01); ***C12N 9/0069*** (2013.01)

(58) **Field of Classification Search**
USPC 435/8
See application file for complete search history.

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(57) **ABSTRACT**

The present invention encompasses modified luciferases, methods for making modified luciferases, and assays utilizing modified luciferases. Modified luciferases of the invention show increased activity over wildtype luciferases and also show increased stability of signal. The present invention also encompasses multiplex assays utilizing multiple luciferases reporters with different emission spectra and different substrates for simultaneous luciferase measurements.

1 Claim, 42 Drawing Sheets

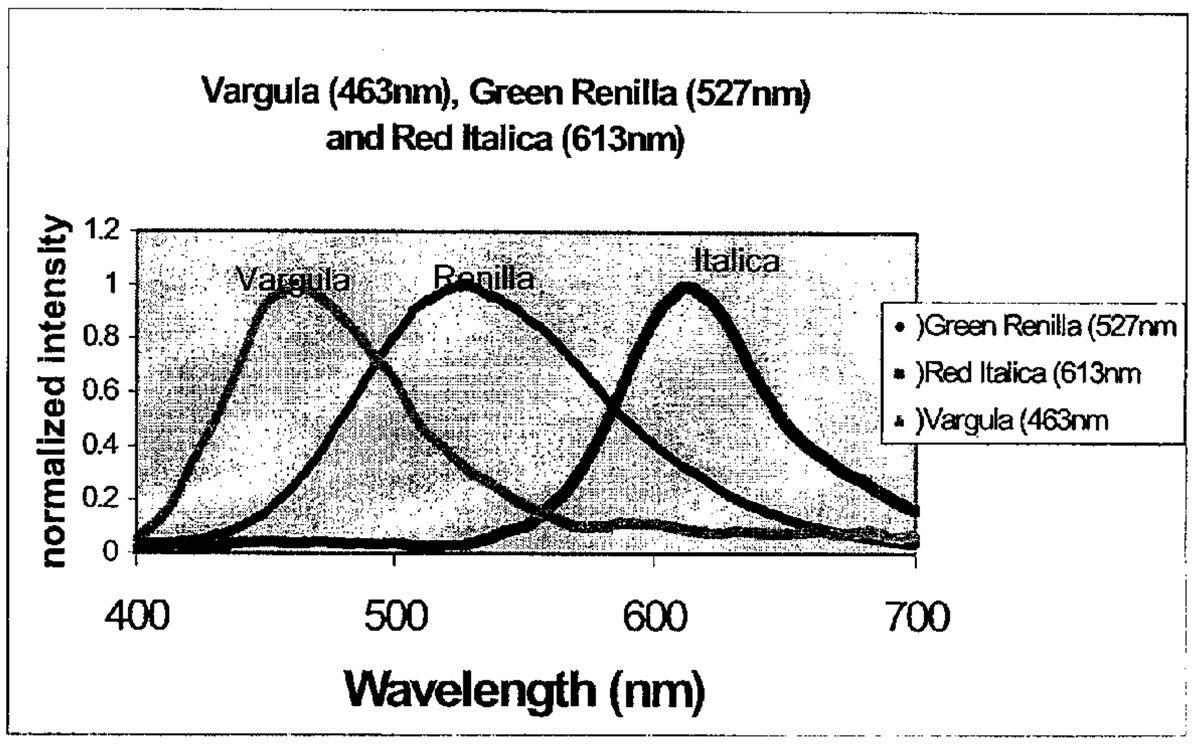

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FIG. 1

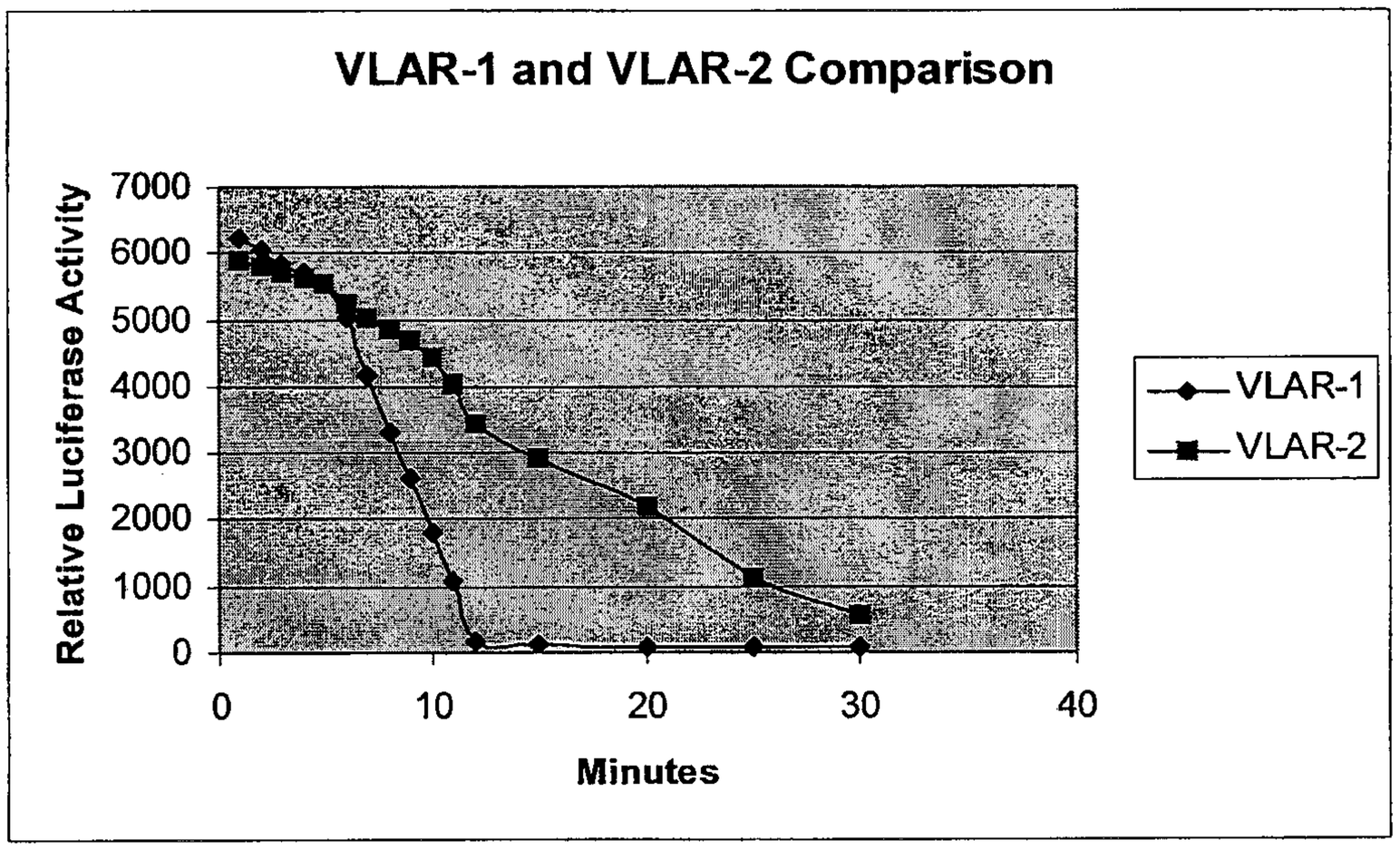
VLAR-1 and VLAR-2 Comparison
Relative Luciferase Activity
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Minutes
VLAR-1
VLAR-2

FIG. 2A. Sample volume 20 µl

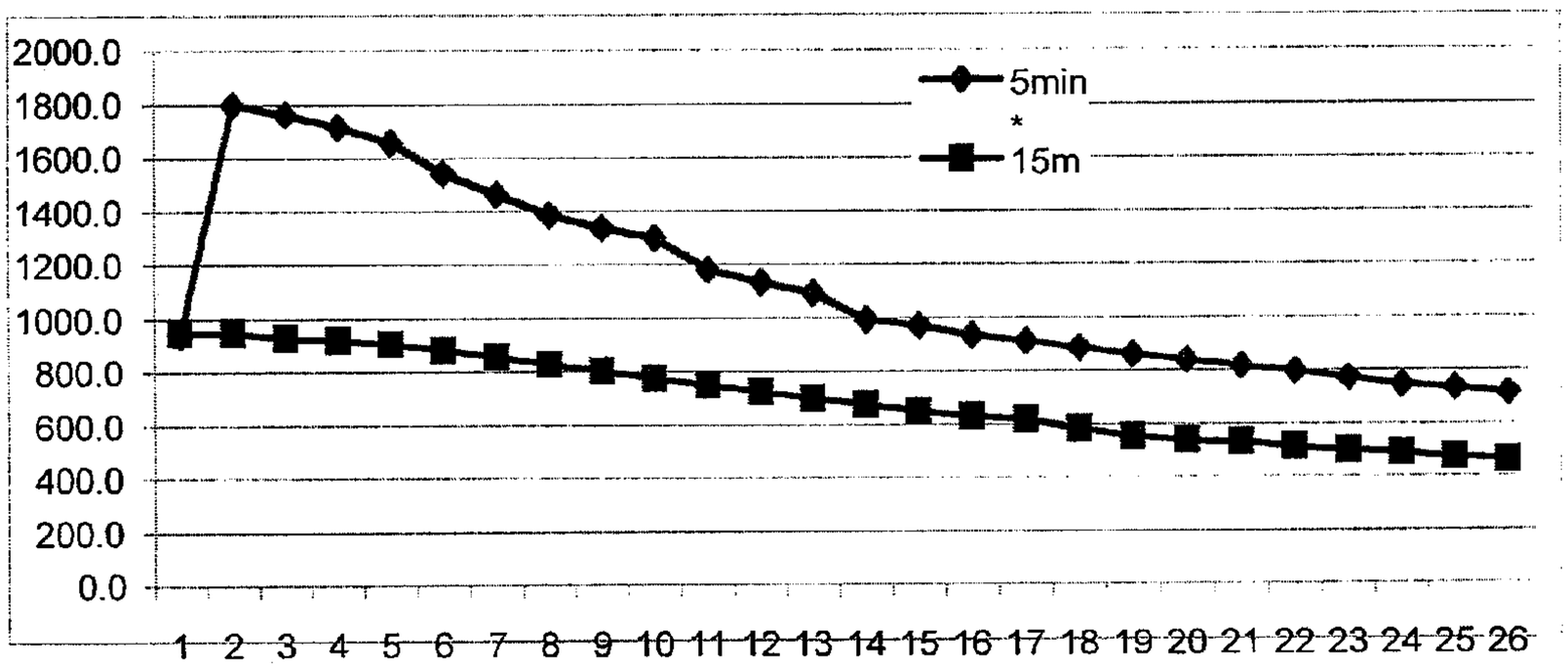

FIG. 2B. Sample volume 5

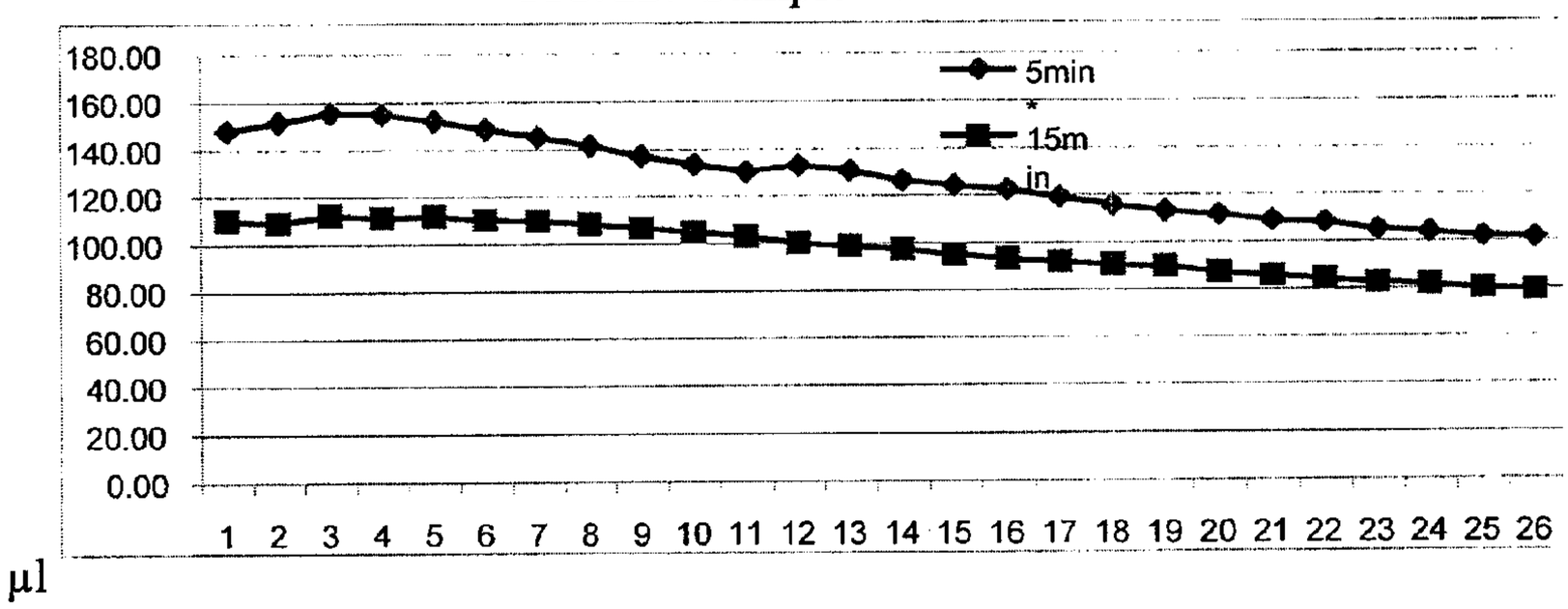

FIG. 3A

pCMV Green Renilla Luciferase plasmid Sequence (SEQ ID NO: 1):

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc
901 gagctcggat ccatgttgtt gaaagttgtg tttgctattg gatgtatcgt agtgcaggct
961 atggcctcaa aagtgtacga tccggagcag cggaagagga tgatcacggg gccccaatgg
1021 tgggcacgat gcaagcagat gaatgtgttg gacagtttca ttaactacta cgacagcgag
1081 aaacacgcgg agaacgcagt gatattcctg cacggcaatg caaccagtag ctatctgtgg
1141 agacacgtgg tgcctcatat tgagccggtc gctagatgca ttattcccga tcttattgga
1201 atggggaaat ccggaaagag tggaaatgga tcatataggc tcctcgatca ttataaatat
1261 ctgactgctt ggtttgaatt gctcaatctg cccaagaaaa tcatctttgt aggacatgat
1321 tggggctccg cccttgcttt tcattatgcc tatgaacacc aggatcggat caaggctatt
1381 gttcacatgg agagcgtggt ggatgtgatt gaatcatgga tgggttggcc ggatatagaa
1441 gaagagctgg cgctgattaa atctgaggag ggcgagaaga tggtactcga aaataacttc
1501 tttgtcgaga cggtactgcc cagtaagatc atgcgcaaac tggagcctga agagtttgcg
1561 gcttacctgg aacccttcaa ggagaaggga gaggtgagga gaccgaccct gtcatggcct
1621 cgggaaattc cgctggtcaa aggagggaag ccagacgtcg tcgccattgt ccggaattac
1681 aacgcttacc tccgcgctag tgacgacctg cctaaactct tcatcgaatc agatcctggt
1741 ttctttagta acgccatcgt cgagggcgcc aagaagtttc caaacaccga atttgttaaa
1801 gtcaaaggac ttcacttcct ccaggaggat gcgcccgatg aaatgggaaa gtatatcaaa
1861 tccttcgtgg agagggtctt gaagaatgag cagaggtcca tctagtctag aaataattct
1921 tactgtcatg ccaagtaaga tgcttttctg tgctgcaata gcaggcatgc tggggatgcg
1981 gtgggctcta tggcttctga ggcggaaaga accagctggg gctctagggg gtatccccac
2041 gcgccctgta gcggcgcatt aagcgcggcg ggtgtggtgg ttacgcgcag cgtgaccgct
2101 acacttgcca gcgccctagc gcccgctcct ttcgctttct tcccttcctt tctcgccacg
2161 ttcgccggct ttccccgtca agctctaaat cggggcatcc ctttagggtt ccgatttagt
2221 gctttacggc acctcgaccc caaaaaactt gattagggtg atggttcacg tagtgggcca
2281 tcgccctgat agacggtttt tcgccctttg acgttggagt ccacgttctt taatagtgga
2341 ctcttgttcc aaactggaac aacactcaac cctatctcgg tctattcttt tgatttataa
2401 gggattttgg ggatttcggc ctattggtta aaaaatgagc tgatttaaca aaaatttaac
2461 gcgaattaat tctgtggaat gtgtgtcagt tagggtgtgg aaagtcccca ggctccccag
2521 gcaggcagaa gtatgcaaag catgcatctc aattagtcag caaccaggtg tggaaagtcc
2581 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata
2641 gtcccgcccc taactccgcc catcccgccc ctaactccgc ccagttccgc ccattctccg
2701 ccccatggct gactaatttt ttttatttat gcagaggccg aggccgcctc tgcctctgag
2761 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttgcaa aaagctcccg
2821 ggagcttgta tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg
2881 attgaacaag atggattgca cgcaggttct ccggccgctt gggtggagag gctattcggc
2941 tatgactggg cacaacagac aatcggctgc tctgatgccg ccgtgttccg gctgtcagcg
3001 caggggcgcc cggttctttt tgtcaagacc gacctgtccg gtgccctgaa tgaactgcag
3061 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc
3121 gacgttgtca ctgaagcggg aagggactgg ctgctattgg gcgaagtgcc ggggcaggat

FIG. 3B

3181 ctcctgtcat ctcaccttgc tcctgccgag aaagtatcca tcatggctga tgcaatgcgg
3241 cggctgcata cgcttgatcc ggctacctgc ccattcgacc accaagcgaa acatcgcatc
3301 gagcgagcac gtactcggat ggaagccggt cttgtcgatc aggatgatct ggacgaagag
3361 catcaggggc tcgcgccagc cgaactgttc gccaggctca aggcgcgcat gcccgacggc
3421 gaggatctcg tcgtgaccca tggcgatgcc tgcttgccga atatcatggt ggaaaatggc
3481 cgcttttctg gattcatcga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata
3541 gcgttggcta cccgtgatat tgctgaagag cttggcggcg aatgggctga ccgcttcctc
3601 gtgctttacg gtatcgccgc tcccgattcg cagcgcatcg ccttctatcg ccttcttgac
3661 gagttcttct gagcgggact ctggggttcg aaatgaccga ccaagcgacg cccaacctgc
3721 catcacgaga tttcgattcc accgccgcct tctatgaaag gttgggcttc ggaatcgttt
3781 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttcttcgccc
3841 accccaactt gtttattgca gcttataatg gttacaaata aagcaatagc atcacaaatt
3901 tcacaaataa agcatttttt tcactgcatt ctagttgtgg tttgtccaaa ctcatcaatg
3961 tatcttatca tgtctgtata ccgtcgacct ctagctagag cttggcgtaa tcatggtcat
4021 agctgtttcc tgtgtgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa
4081 gcataaagtg taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc
4141 gctcactgcc cgctttccag tcgggaaacc tgtcgtgcca gctgcattaa tgaatcggcc
4201 aacgcgcggg gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg ctcactgact
4261 cgctgcgctc ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag gcggtaatac
4321 ggttatccac agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa
4381 aggccaggaa ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgcccccctg
4441 acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa
4501 gataccaggc gtttcccccct ggaagctccc tcgtgcgctc tcctgttccg accctgccgc
4561 ttaccggata cctgtccgcc tttctccctt cgggaagcgt ggcgctttct caatgctcac
4621 gctgtaggta tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac
4681 cccccgttca gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg
4741 taagacacga cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt
4801 atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagga
4861 cagtatttgg tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct
4921 cttgatccgg caaacaaacc accgctggta gcggtggttt ttttgtttgc aagcagcaga
4981 ttacgcgcag aaaaaaagga tctcaagaag atcctttgat cttttctacg gggtctgacg
5041 ctcagtggaa cgaaaactca cgttaaggga ttttggtcat gagattatca aaaaggatct
5101 tcacctagat ccttttaaat taaaaatgaa gttttaaatc aatctaaagt atatatgagt
5161 aaacttggtc tgacagttac caatgcttaa tcagtgaggc acctatctca gcgatctgtc
5221 tatttcgttc atccatagtt gcctgactcc ccgtcgtgta gataactacg atacgggagg
5281 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag
5341 atttatcagc aataaaccag ccagccggaa gggccgagcg cagaagtggt cctgcaactt
5401 tatccgcctc catccagtct attaattgtt gccgggaagc tagagtaagt agttcgccag
5461 ttaatagttt gcgcaacgtt gttgccattg ctacaggcat cgtggtgtca cgctcgtcgt
5521 ttggtatggc ttcattcagc tccggttccc aacgatcaag gcgagttaca tgatccccca
5581 tgttgtgcaa aaaagcggtt agctccttcg gtcctccgat cgttgtcaga agtaagttgg
5641 ccgcagtgtt atcactcatg gttatggcag cactgcataa ttctcttact gtcatgccat
5701 ccgtaagatg cttttctgtg actggtgagt actcaaccaa gtcattctga gaatagtgta
5761 tgcggcgacc gagttgctct tgcccggcgt caatacggga taataccgcg ccacatagca
5821 gaactttaaa agtgctcatc attggaaaac gttcttcggg gcgaaaactc tcaaggatct
5881 taccgctgtt gagatccagt tcgatgtaac ccactcgtgc acccaactga tcttcagcat
5941 cttttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa
6001 agggaataag ggcgacacgg aaatgttgaa tactcatact cttccttttt caatattatt
6061 gaagcattta tcagggttat tgtctcatga gcggatacat atttgaatgt atttagaaaa
6121 ataaacaaat aggggttccg cgcacatttc cccgaaaagt gccacctgac gtc

FIG. 4A

Modified red firefly luciferase with secretory signal (SEQ ID NO: 2)

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc
901 gagctcggat cc atggccttcctgtggctgctgtcctgctgggccctgctgggcaccaccttcggc
961 tacccgatcg aggagggctc tgccggcatc caattgcaca agtacatgca acaatacgcc
1021 aagctcggcg ccatcgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081 tacttcgaca tcacgtgcag actcgccgag gctatgaaga actacggcat gaagccagaa
1141 ggacacatcg ctctctgtag cgagaactgc gaagagttct tcattcctgt tctggctggt
1201 ctttacatcg gagttacagt cgcgccaact aacgaaattt atacacttag agagctgaac
1261 cacagtctgg ggatagccca acctactatc gtattctcta gcaggaaggg cctgcccaaa
1321 gtgcttgagg tgcagaagac cgtgacttgc atcaaaacca ttgtcatcct ggacagtaag
1381 gtcaacttcg gcggttatga ctgcgtagag accttcatta agaaacacgt cgagctgggc
1441 tttcctgcca cctcatttgt gcccatcgac gtcaaagacc ggaagcacca cattgctctg
1501 cttatgaact cttccggttc cacagggctg cccaaaggag tagagatcac tcacgaggcc
1561 ctggtcacga gattctctca cgctaaggac cctatatacg gcaatcaggt ggccccaggt
1621 accgctatcc tgactgtcgt gcctttccac cacggcttcg gaatgttcac tactttgggc
1681 tactttgcct gcggttaccg gattgtcatg cttactaagt tcgacgagga gcttttcctg
1741 cgcacacttc aggattacaa gtgcactaca gtaatcctgg tgccgacact gttcgcaatt
1801 cttaataggt ctgagctcct tgataagttt gacctctcta acctgactga aatagccagc
1861 ggtggtgctc cacttgccaa ggagatcggc gaggctgttg caagaagatt caacctccca
1921 ggcgtccggc agggatatgg actcaccgag actaccagtg cctttatcat cactcctaag
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgttcaaggt gaagattatt
2041 gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101 ccatctctta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca cttctttata
2221 gtcgaccgcc tgaaatctct cattaagtat aaaggatacc aagtgccacc agctgaactg
2281 gagtctgtgc tcctgcaaca ccctaacatt agagatgctg gtgtggccgg ggttcccgac
2341 agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401 gagaaagaaa tcgtagacta tgtaaactcc caggtggtca accacaagcg gctgaggggc
2461 ggcgtgcggt tcgtagatga agtccccaag gggctcacag gaaagatcga cgcgaaagtt
2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctagatctag aaataattct
2581 tactgtcatg ccaagtaaga tgcttttctg tgctgcaata gcaggcatgc tggggatgcg
2641 gtgggctcta tggcttctga ggcggaaaga accagctggg gctctagggg gtatccccac
2701 gcgccctgta gcggcgcatt aagcgcggcg ggtgtggtgg ttacgcgcag cgtgaccgct
2761 acacttgcca gcgccctagc gcccgctcct ttcgctttct tcccttcctt tctcgccacg
2821 ttcgccggct ttccccgtca agctctaaat cggggcatcc ctttagggtt ccgatttagt
2881 gctttacggc acctcgaccc caaaaaactt gattagggtg atggttcacg tagtgggcca
2941 tcgccctgat agacggtttt tcgcccttg acgttggagt ccacgttctt taatagtgga
3001 ctcttgttcc aaactggaac aacactcaac cctatctcgg tctattcttt tgatttataa
3061 gggattttgg ggatttcggc ctattggtta aaaaatgagc tgatttaaca aaaatttaac
3121 gcgaattaat tctgtggaat gtgtgtcagt tagggtgtgg aaagtcccca ggctccccag
3181 gcaggcagaa gtatgcaaag catgcatctc aattagtcag caaccaggtg tggaaagtcc

FIG. 4B

3241 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata
3301 gtcccgcccc taactccgcc catcccgccc ctaactccgc ccagttccgc ccattctccg
3361 ccccatggct gactaatttt ttttatttat gcagaggccg aggccgcctc tgcctctgag
3421 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttgcaa aaagctcccg
3481 ggagcttgta tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg
3541 attgaacaag atggattgca cgcaggttct ccggccgctt gggtggagag gctattcggc
3601 tatgactggg cacaacagac aatcggctgc tctgatgccg ccgtgttccg gctgtcagcg
3661 caggggcgcc cggttctttt tgtcaagacc gacctgtccg gtgccctgaa tgaactgcag
3721 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc
3781 gacgttgtca ctgaagcggg aagggactgg ctgctattgg gcgaagtgcc ggggcaggat
3841 ctcctgtcat ctcaccttgc tcctgccgag aaagtatcca tcatggctga tgcaatgcgg
3901 cggctgcata cgcttgatcc ggctacctgc ccattcgacc accaagcgaa acatcgcatc
3961 gagcgagcac gtactcggat ggaagccggt cttgtcgatc aggatgatct ggacgaagag
4021 catcaggggc tcgcgccagc cgaactgttc gccaggctca aggcgcgcat gcccgacggc
4081 gaggatctcg tcgtgaccca tggcgatgcc tgcttgccga atatcatggt ggaaaatggc
4141 cgcttttctg gattcatcga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata
4201 gcgttggcta cccgtgatat tgctgaagag cttggcggcg aatgggctga ccgcttcctc
4261 gtgctttacg gtatcgccgc tcccgattcg cagcgcatcg ccttctatcg ccttcttgac
4321 gagttcttct gagcgggact ctggggttcg aaatgaccga ccaagcgacg cccaacctgc
4381 catcacgaga tttcgattcc accgccgcct tctatgaaag gttgggcttc ggaatcgttt
4441 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttcttcgccc
4501 accccaactt gtttattgca gcttataatg gttacaaata aagcaatagc atcacaaatt
4561 tcacaaataa agcatttttt tcactgcatt ctagttgtgg tttgtccaaa ctcatcaatg
4621 tatcttatca tgtctgtata ccgtcgacct ctagctagag cttggcgtaa tcatggtcat
4681 agctgtttcc tgtgtgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa
4741 gcataaagtg taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc
4801 gctcactgcc cgctttccag tcgggaaacc tgtcgtgcca gctgcattaa tgaatcggcc
4861 aacgcgcggg gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg ctcactgact
4921 cgctgcgctc ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag gcggtaatac
4981 ggttatccac agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa
5041 aggccaggaa ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgccccctg
5101 acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa
5161 gataccaggc gtttcccccc ggaagctccc tcgtgcgctc tcctgttccg accctgccgc
5221 ttaccggata cctgtccgcc tttctccctt cgggaagcgt ggcgctttct caatgctcac
5281 gctgtaggta tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac
5341 cccccgttca gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg
5401 taagacacga cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt
5461 atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagga
5521 cagtatttgg tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct
5581 cttgatccgg caaacaaacc accgctggta gcggtggttt ttttgtttgc aagcagcaga
5641 ttacgcgcag aaaaaaagga tctcaagaag atcctttgat cttttctacg gggtctgacg
5701 ctcagtggaa cgaaaactca cgttaaggga ttttggtcat gagattatca aaaaggatct
5761 tcacctagat ccttttaaat taaaaatgaa gttttaaatc aatctaaagt atatatgagt
5821 aaacttggtc tgacagttac caatgcttaa tcagtgaggc acctatctca gcgatctgtc
5881 tatttcgttc atccatagtt gcctgactcc ccgtcgtgta gataactacg atacgggagg
5941 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag
6001 atttatcagc aataaaccag ccagccggaa gggccgagcg cagaagtggt cctgcaactt
6061 tatccgcctc catccagtct attaattgtt gccgggaagc tagagtaagt agttcgccag
6121 ttaatagttt gcgcaacgtt gttgccattg ctacaggcat cgtggtgtca cgctcgtcgt
6181 ttggtatggc ttcattcagc tccggttccc aacgatcaag gcgagttaca tgatccccca
6241 tgttgtgcaa aaaagcggtt agctccttcg gtcctccgat cgttgtcaga agtaagttgg
6301 ccgcagtgtt atcactcatg gttatggcag cactgcataa ttctcttact gtcatgccat
6361 ccgtaagatg cttttctgtg actggtgagt actcaaccaa gtcattctga gaatagtgta
6421 tgcggcgacc gagttgctct tgcccggcgt caatacggga taataccgcg ccacatagca
6481 gaactttaaa agtgctcatc attggaaaac gttcttcggg gcgaaaactc tcaaggatct
6541 taccgctgtt gagatccagt tcgatgtaac ccactcgtgc acccaactga tcttcagcat

FIG. 4C

6601 cttttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa
6661 agggaataag ggcgacacgg aaatgttgaa tactcatact cttccttttt caatattatt
6721 gaagcattta tcagggttat tgtctcatga gcggatacat atttgaatgt atttagaaaa
6781 ataaacaaat aggggttccg cgcacatttc cccgaaaagt gccacctgac gtc

FIG. 5

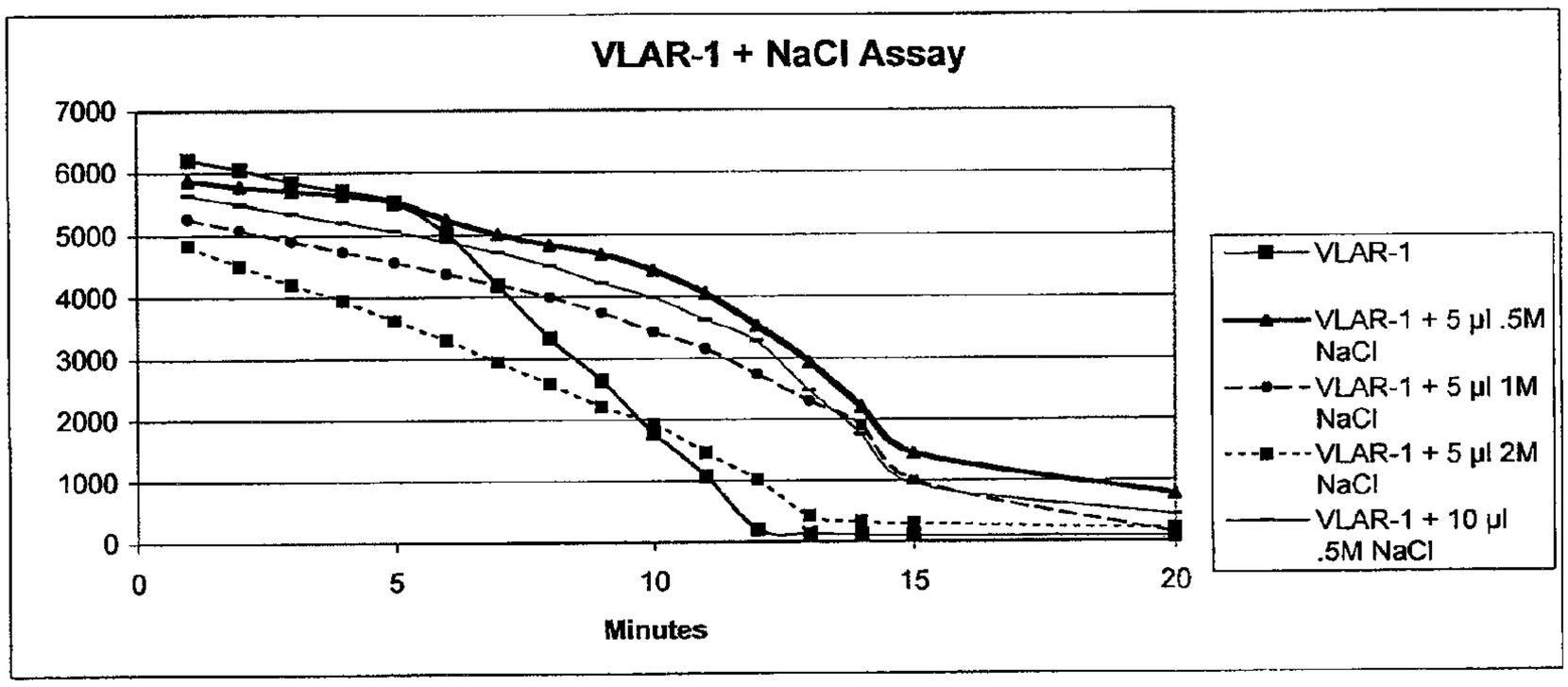
VLAR-1 + NaCl Assay
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
Minutes
VLAR-1
VLAR-1 + 5 µl .5M NaCl
VLAR-1 + 5 µl 1M NaCl
VLAR-1 + 5 µl 2M NaCl
VLAR-1 + 10 µl .5M NaCl

FIG. 6

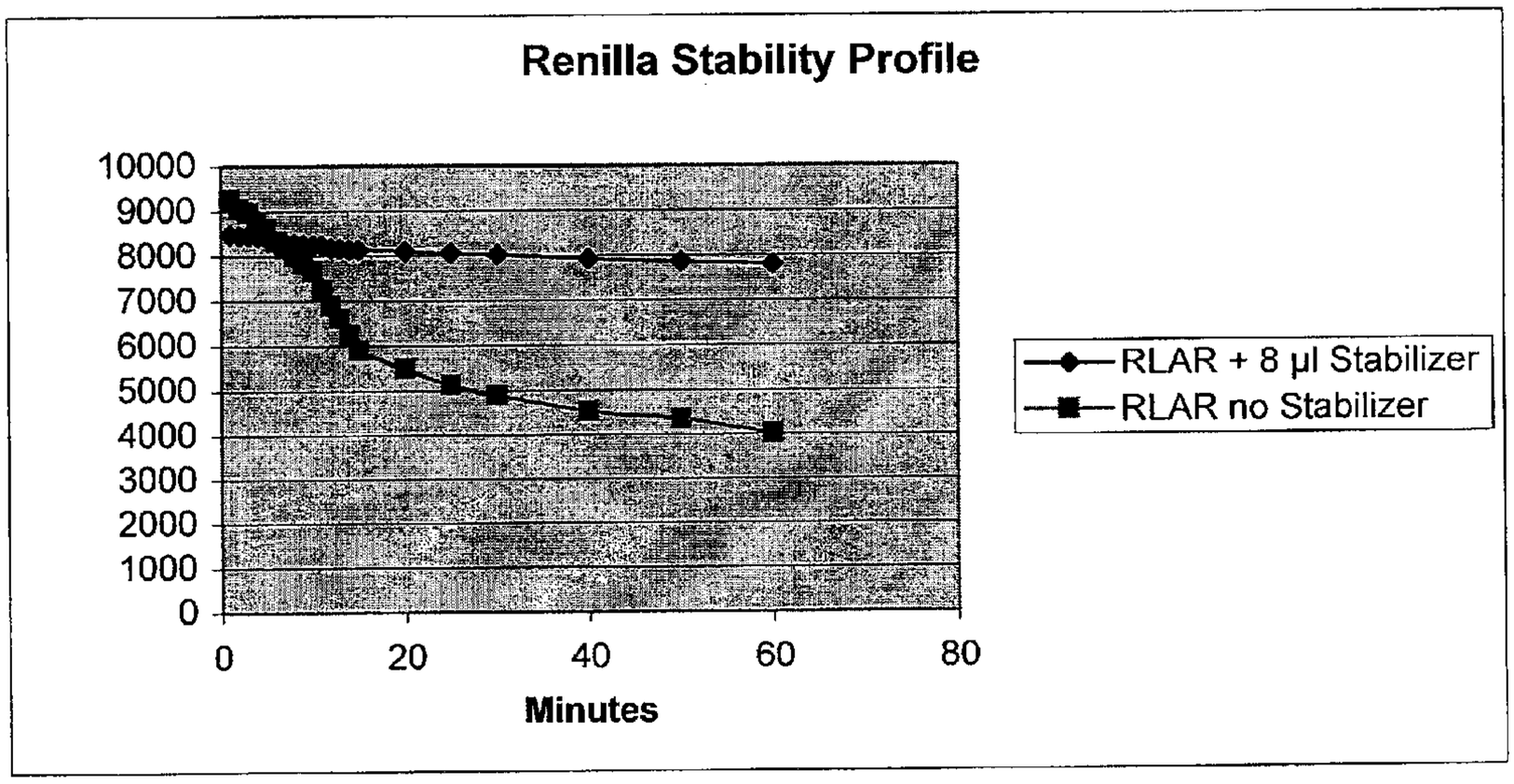
Renilla Stability Profile
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
Minutes
RLAR + 8 µl Stabilizer
RLAR no Stabilizer

FIG. 7

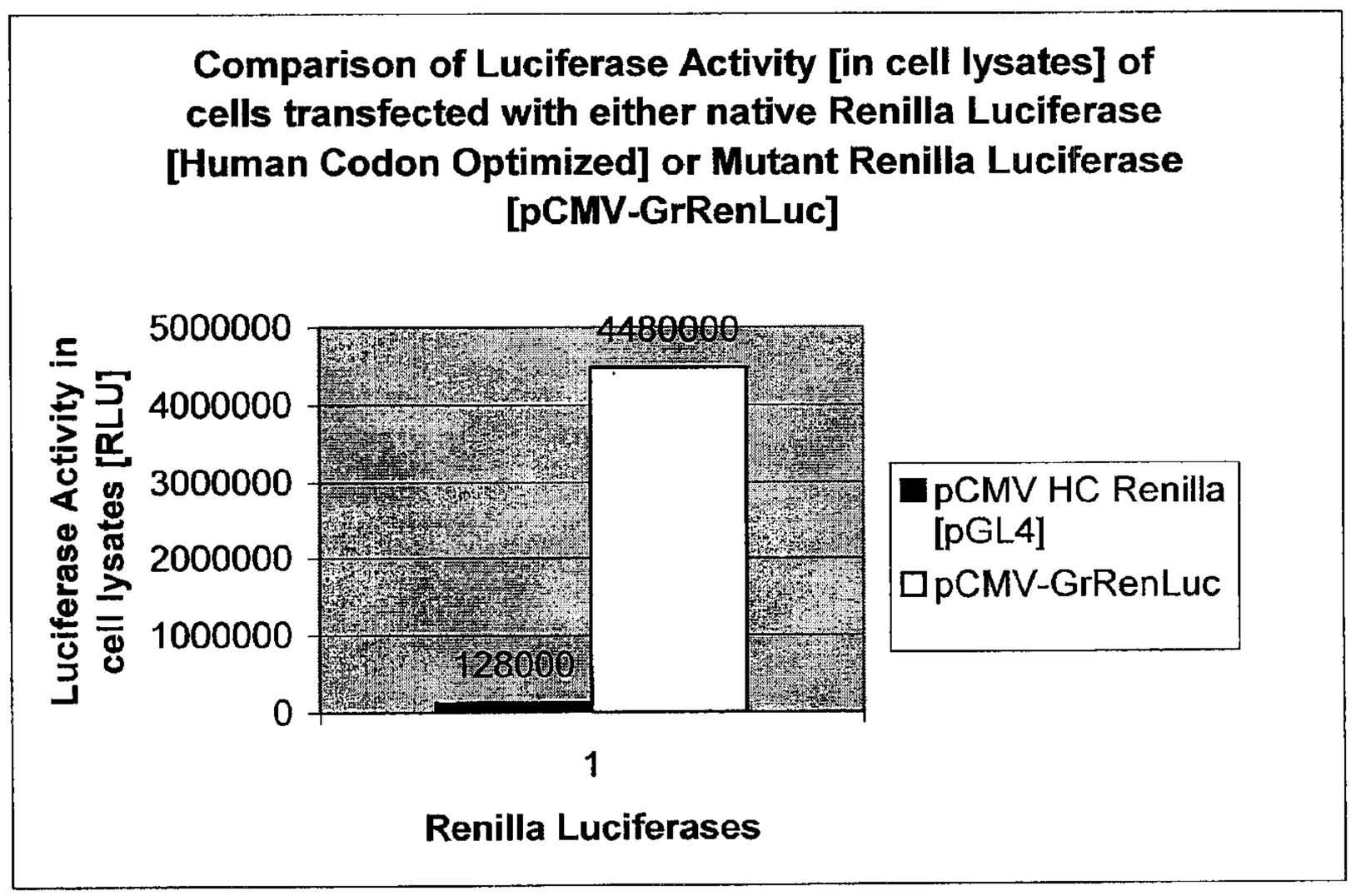
Comparison of Luciferase Activity [in cell lysates] of cells transfected with either native Renilla Luciferase [Human Codon Optimized] or Mutant Renilla Luciferase [pCMV-GrRenLuc]
Luciferase Activity in cell lysates [RLU]
5000000
4000000
3000000
2000000
1000000
0
4480000
128000
1
Renilla Luciferases
pCMV HC Renilla [pGL4]
pCMV-GrRenLuc

FIG. 8

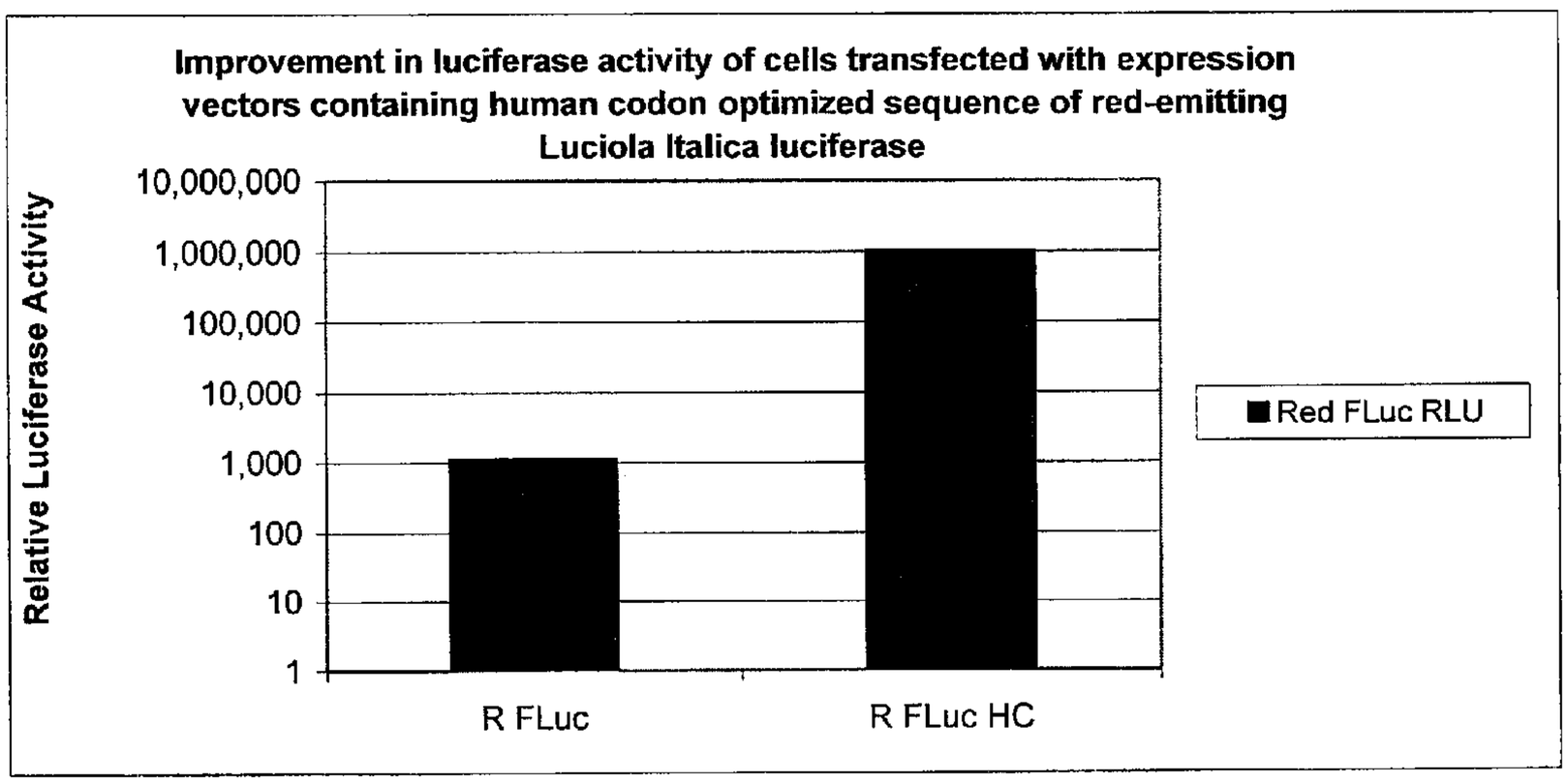
Improvement in luciferase activity of cells transfected with expression vectors containing human codon optimized sequence of red-emitting Luciola Italica luciferase
Relative Luciferase Activity
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
R FLuc
R FLuc HC
Red FLuc RLU

FIG. 9

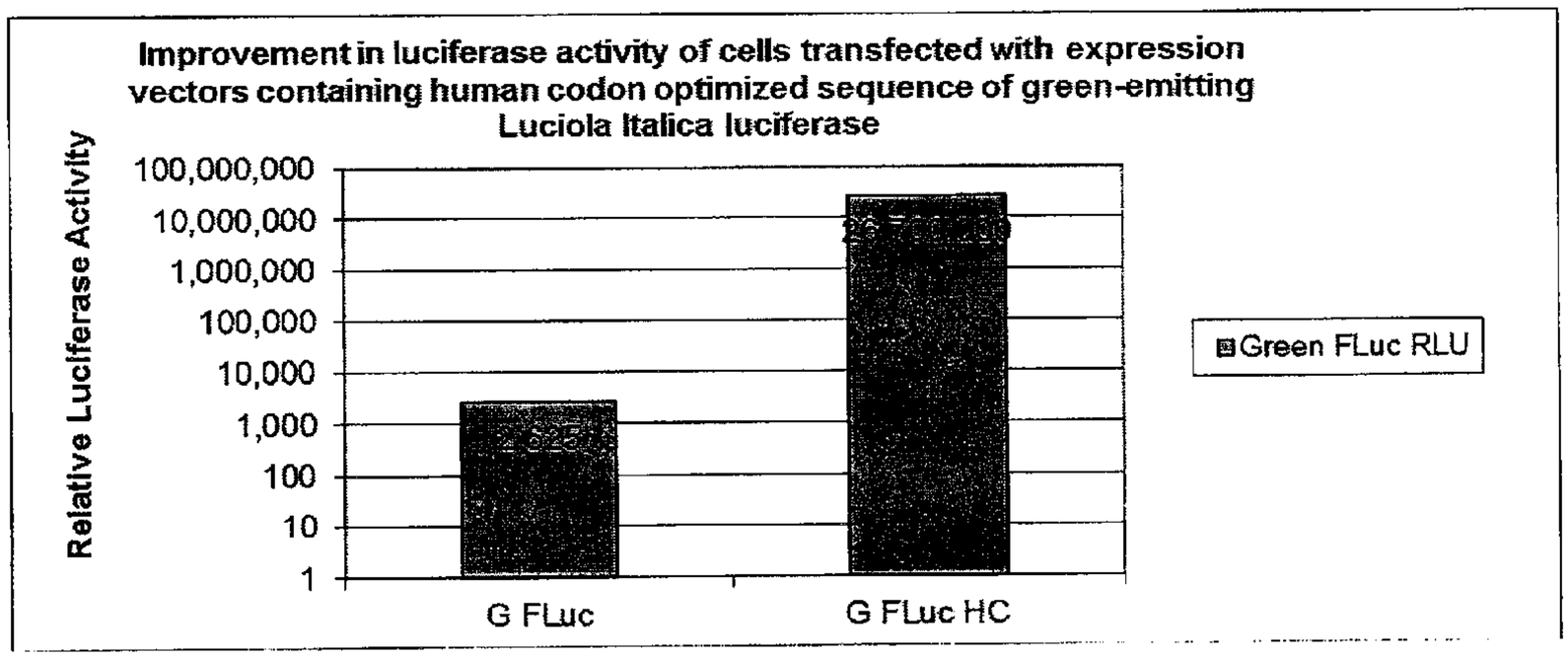
Improvement in luciferase activity of cells transfected with expression vectors containing human codon optimized sequence of green-emitting Luciola Italica luciferase
Relative Luciferase Activity
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
G FLuc
G FLuc HC
Green FLuc RLU

FIG. 10

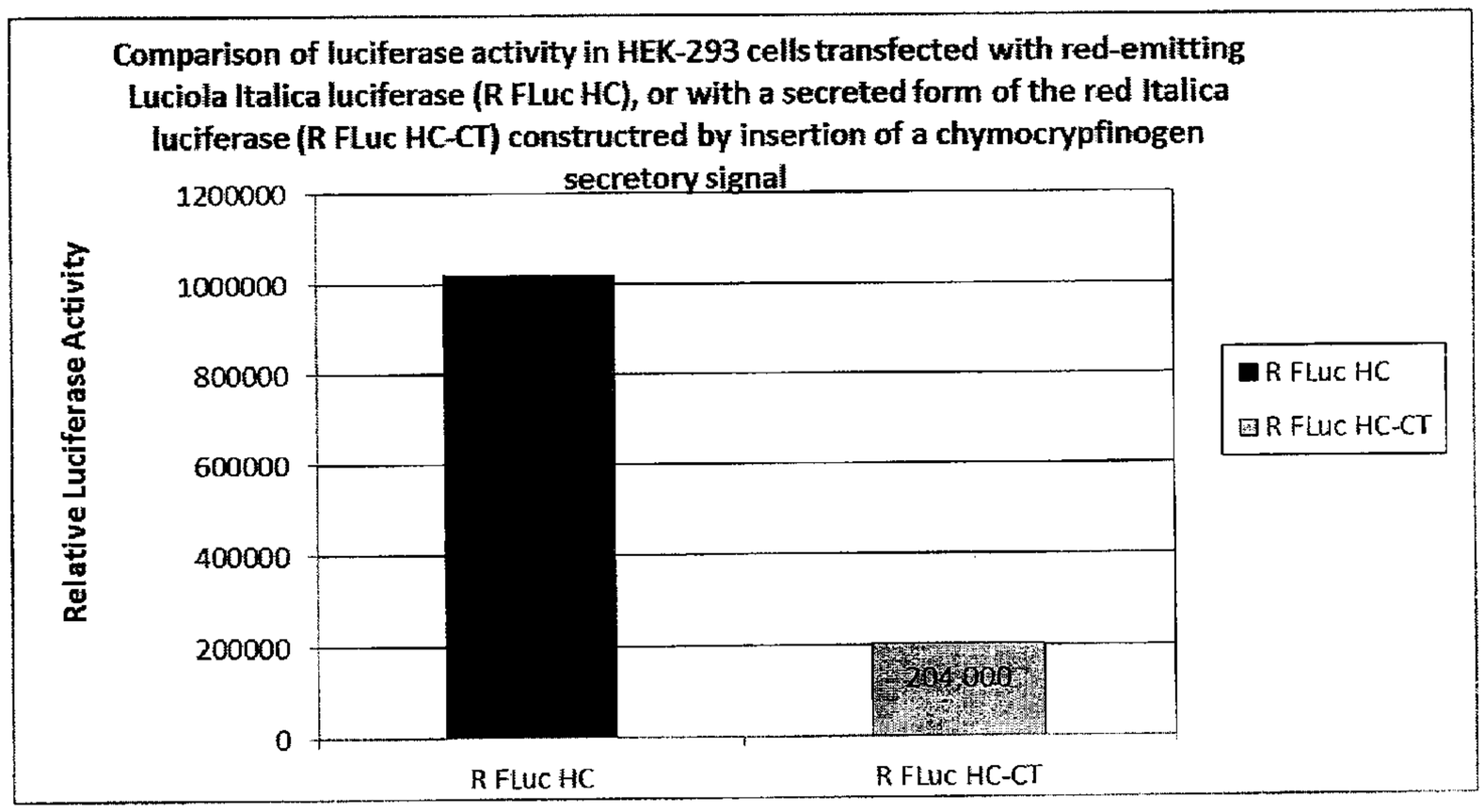
Comparison of luciferase activity in HEK-293 cells transfected with red-emitting Luciola Italica luciferase (R FLuc HC), or with a secreted form of the red Italica luciferase (R FLuc HC-CT) constructred by insertion of a chymocrypfinogen secretory signal
Relative Luciferase Activity
1200000
1000000
800000
600000
400000
200000
0
~ 204 000
R FLuc HC
R FLuc HC-CT
R FLuc HC
R FLuc HC-CT

FIG. 11

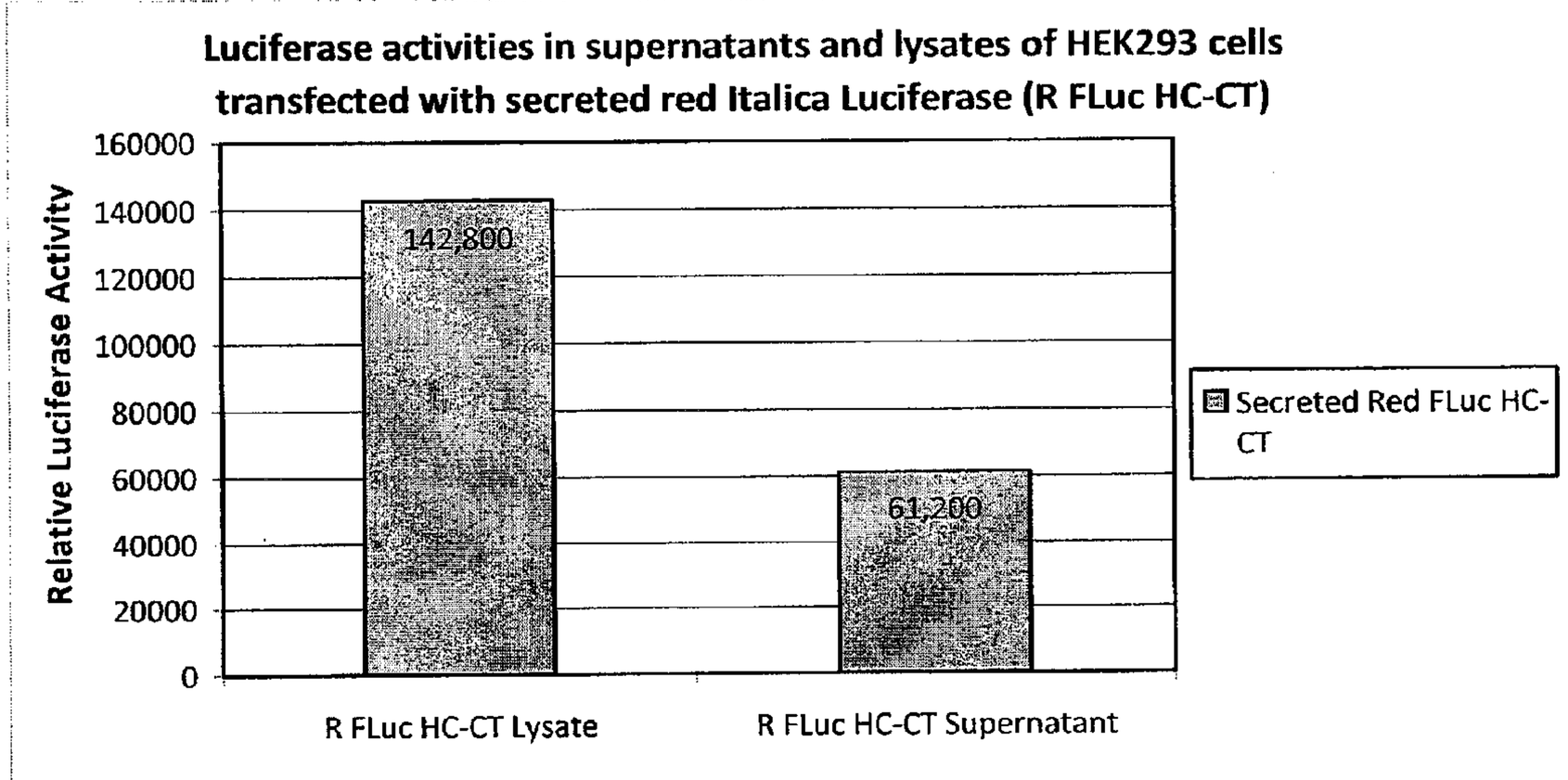
Luciferase activities in supernatants and lysates of HEK293 cells transfected with secreted red Italica Luciferase (R FLuc HC-CT)
Relative Luciferase Activity
160000
140000
120000
100000
80000
60000
40000
20000
0
142,800
61,200
R FLuc HC-CT Lysate
R FLuc HC-CT Supernatant
Secreted Red FLuc HC-CT

FIG. 12A

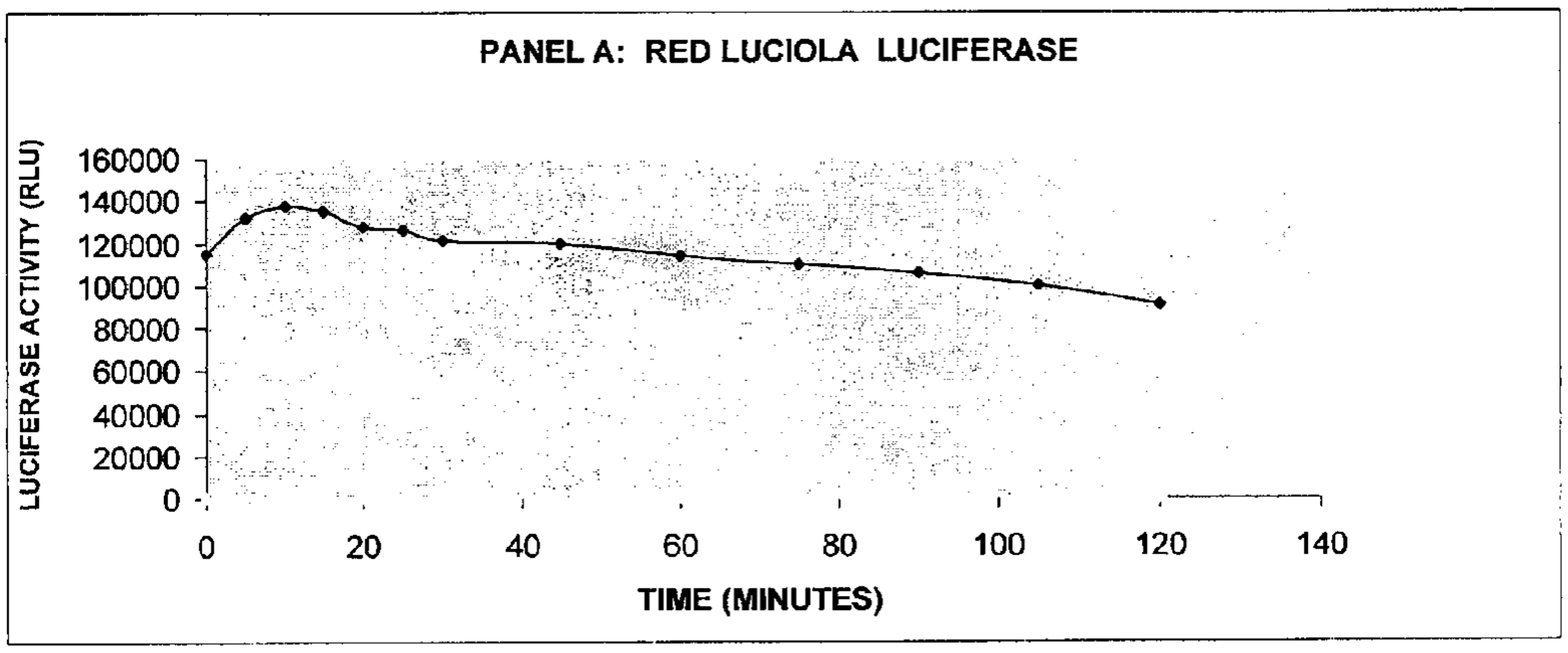

FIG. 12B

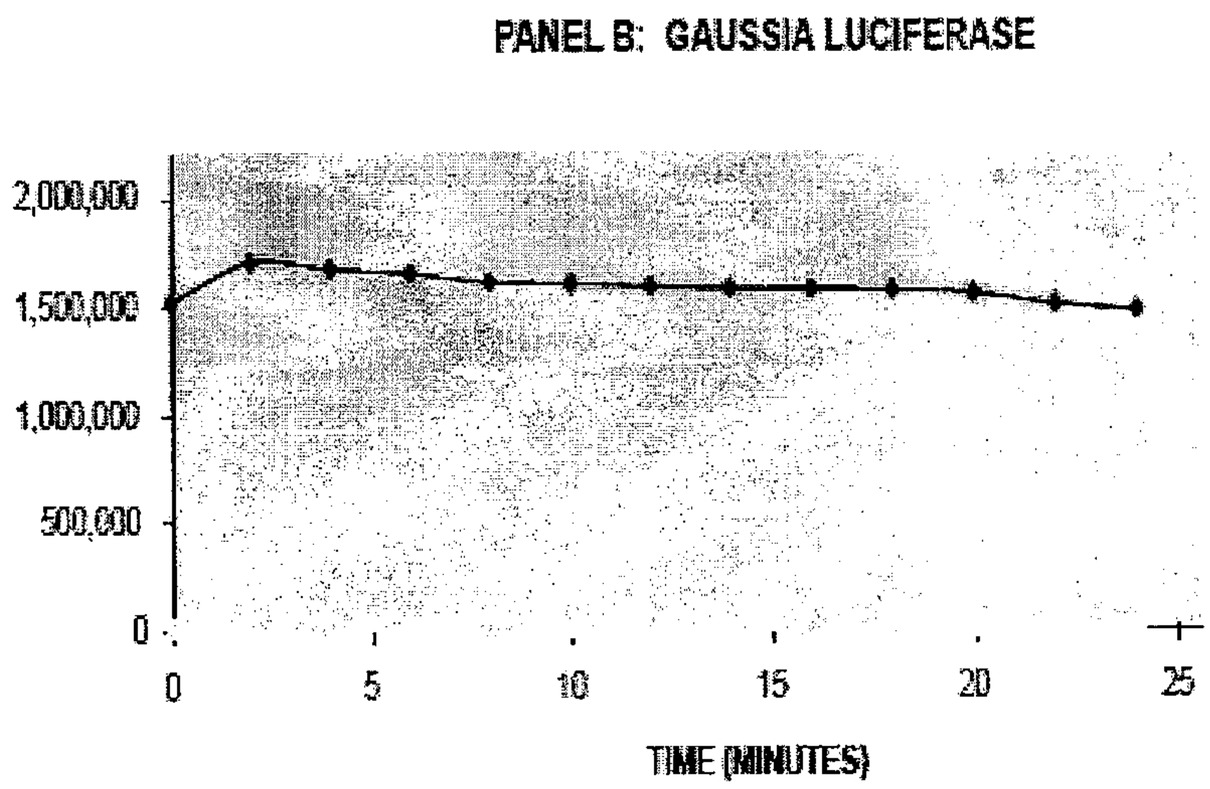

FIG. 12C

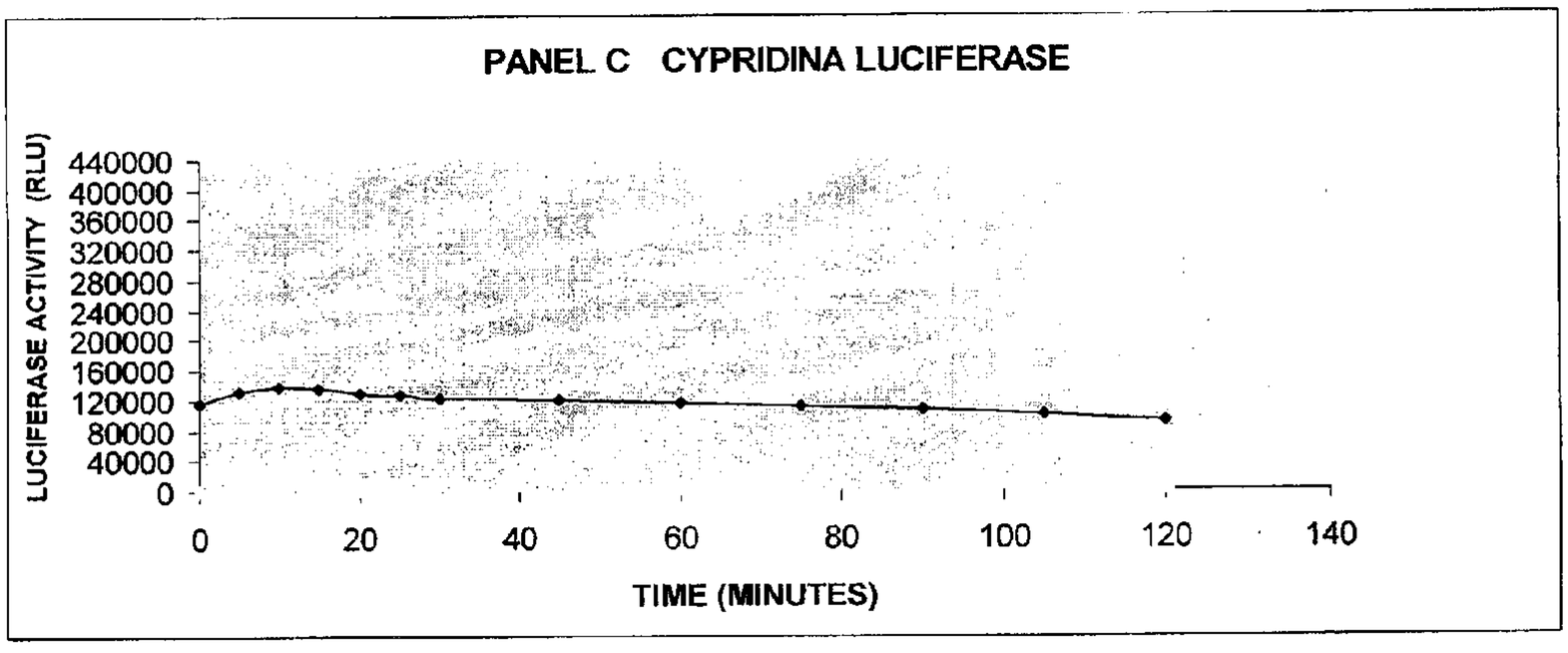

FIG. 12D

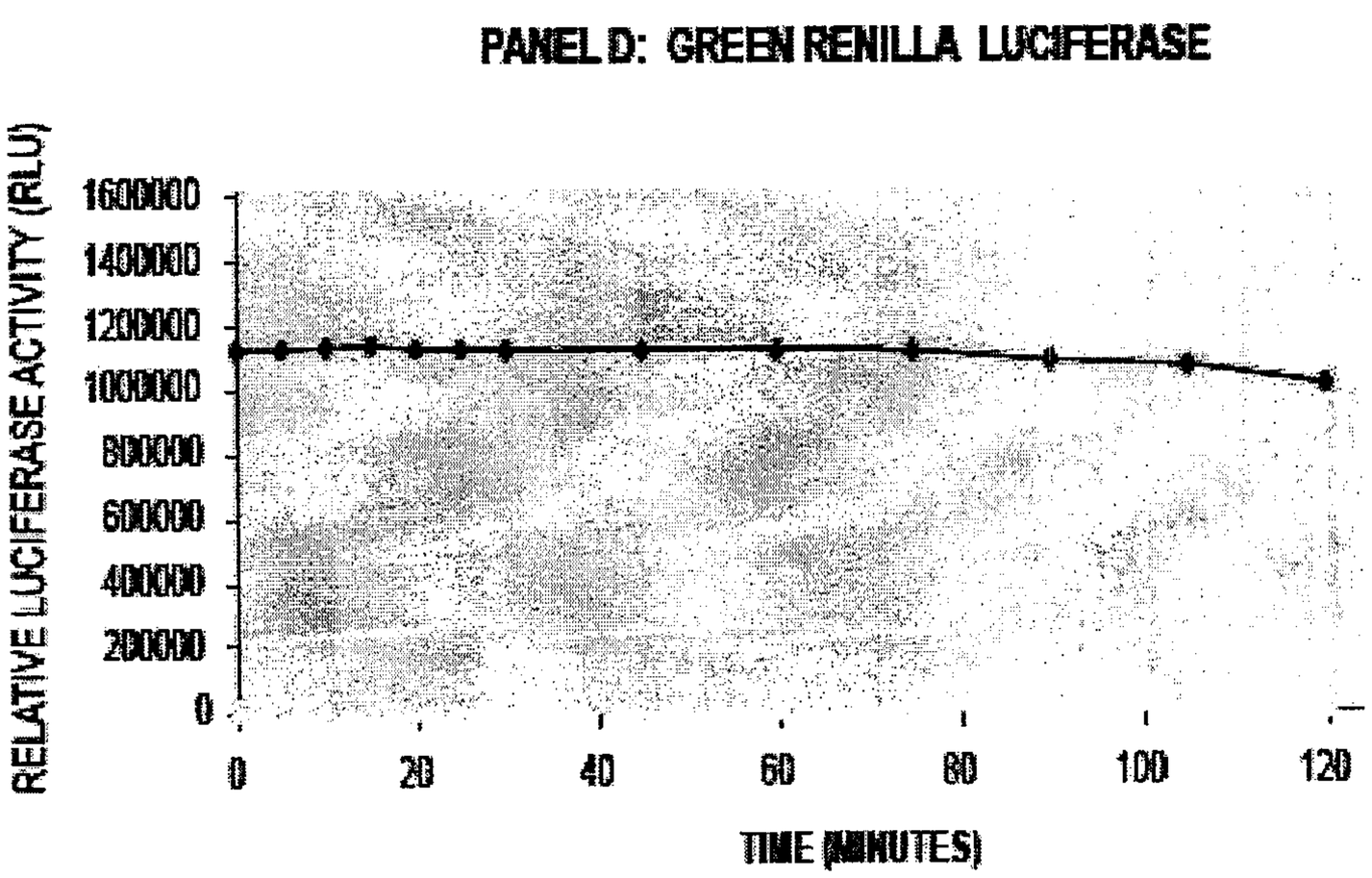
PANEL D: GREEN RENILLA LUCIFERASE
RELATIVE LUCIFERASE ACTIVITY (RLU)
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0
20
40
60
80
100
120
TIME (MINUTES)

FIG. 13A

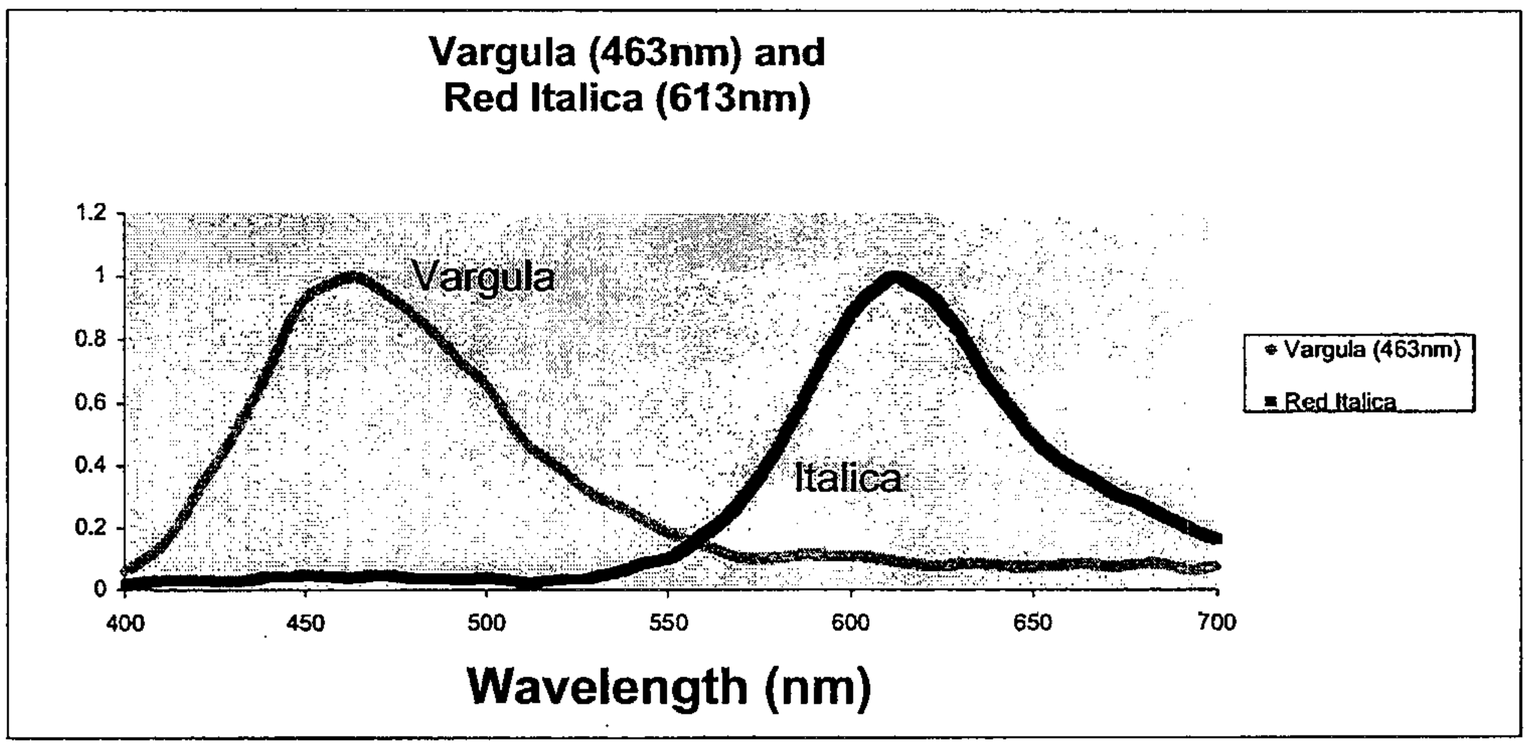

FIG. 13B

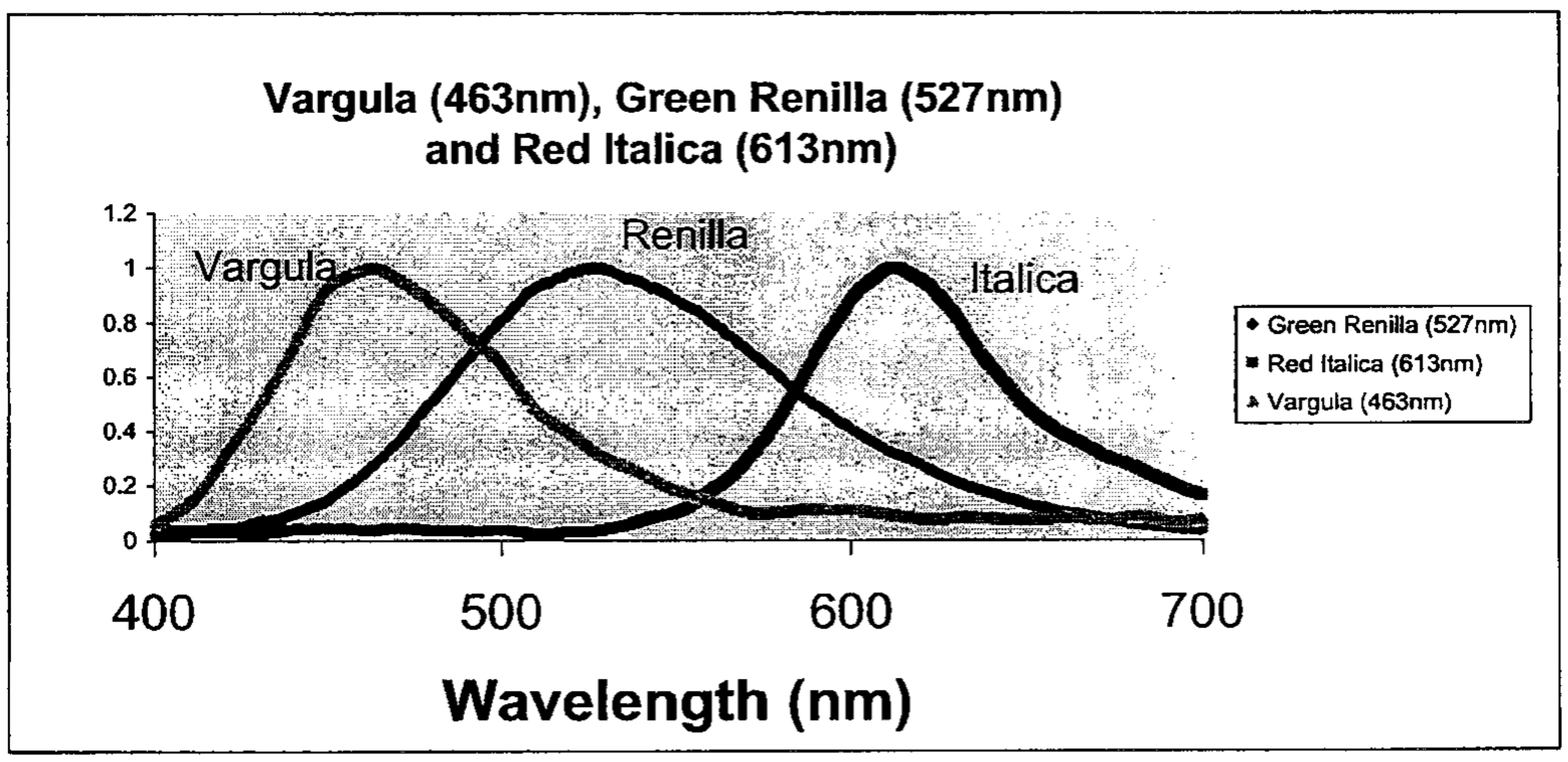

FIG. 14

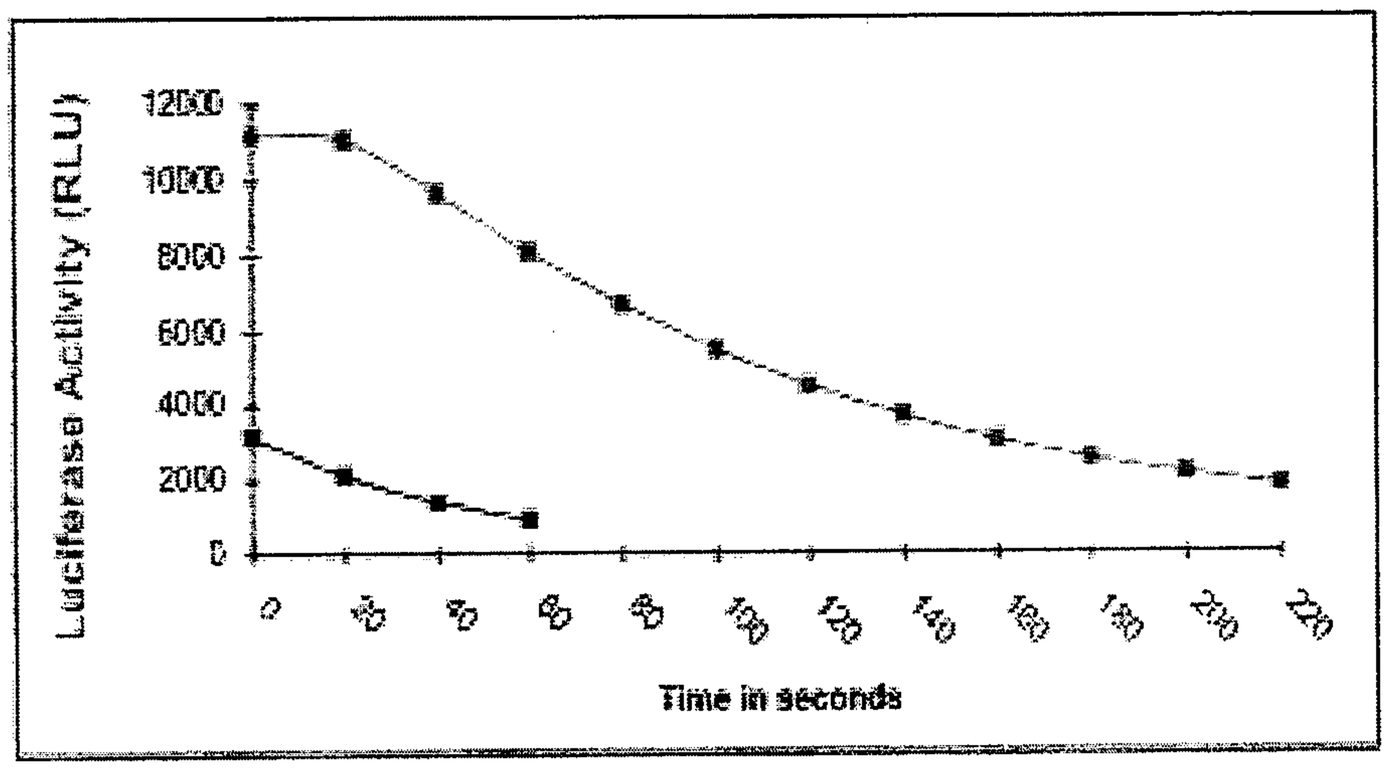
Luciferase Activity (RLU)
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
120
140
160
180
200
220
Time in seconds

FIG. 15A

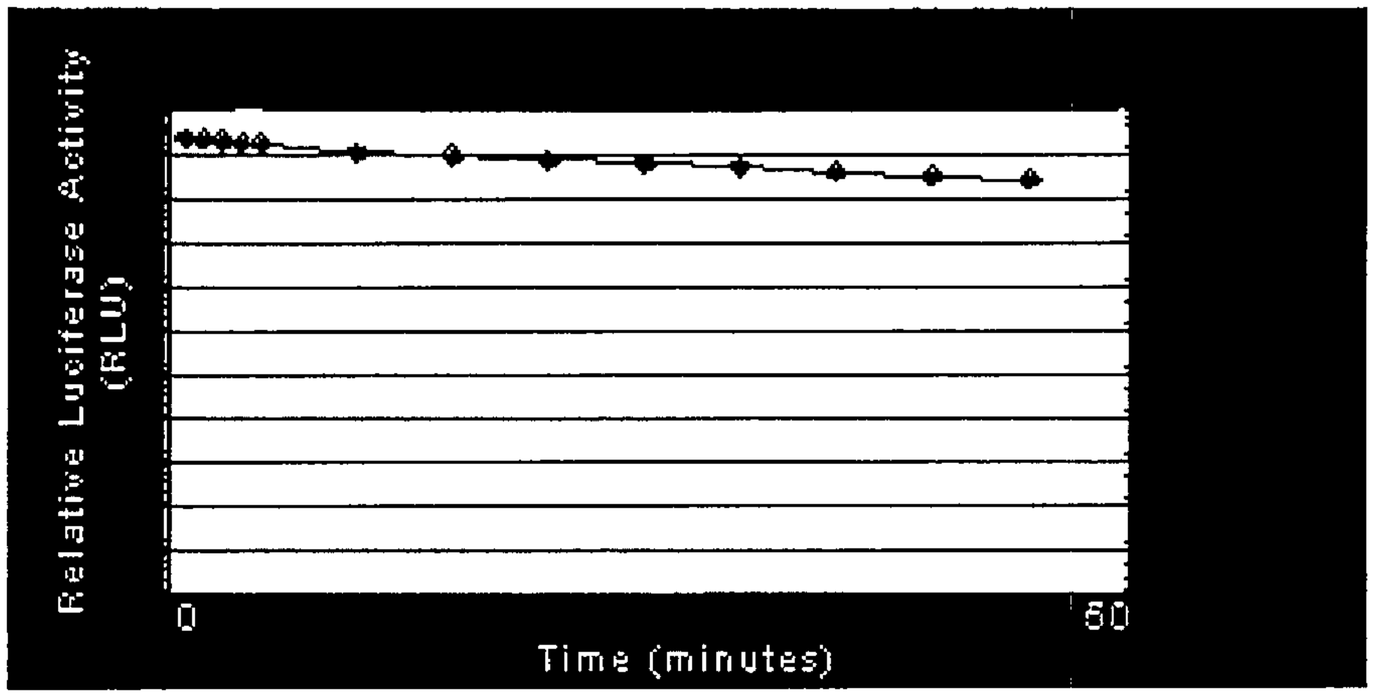

FIG. 15B

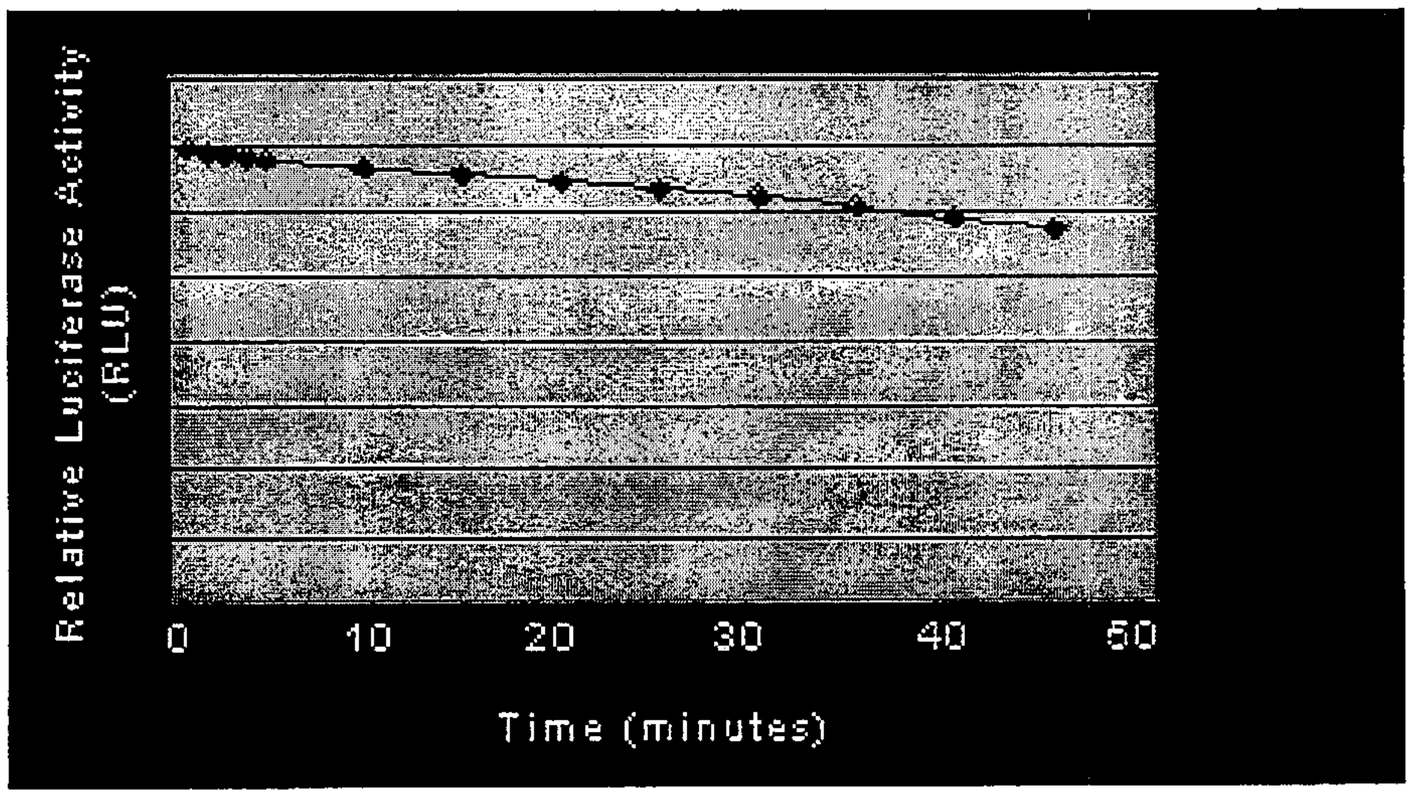

FIG. 16

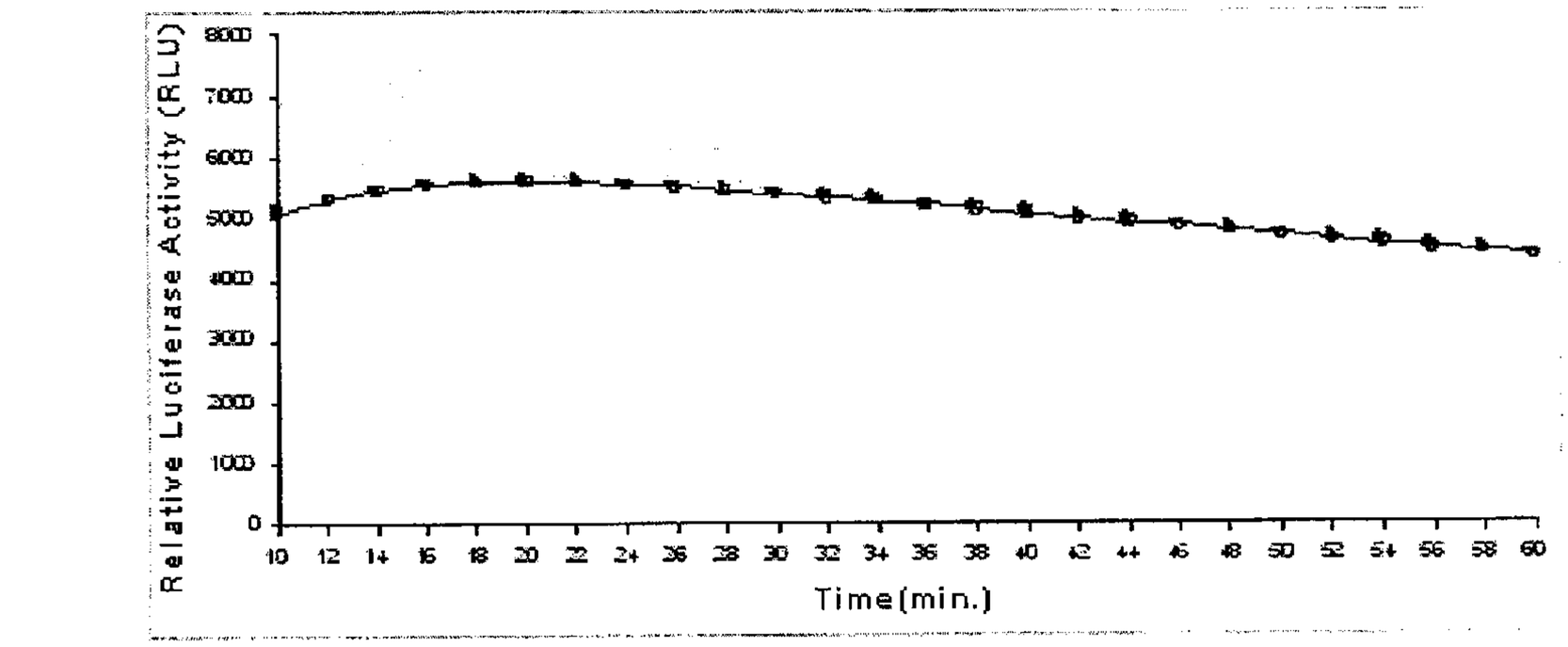
Firefly Luciferase Assay reagent (FLAR-1)
Relative Luciferase Activity (RLU)
8000
7000
6000
5000
4000
3000
2000
1000
0
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
Time(min.)

FIG. 17

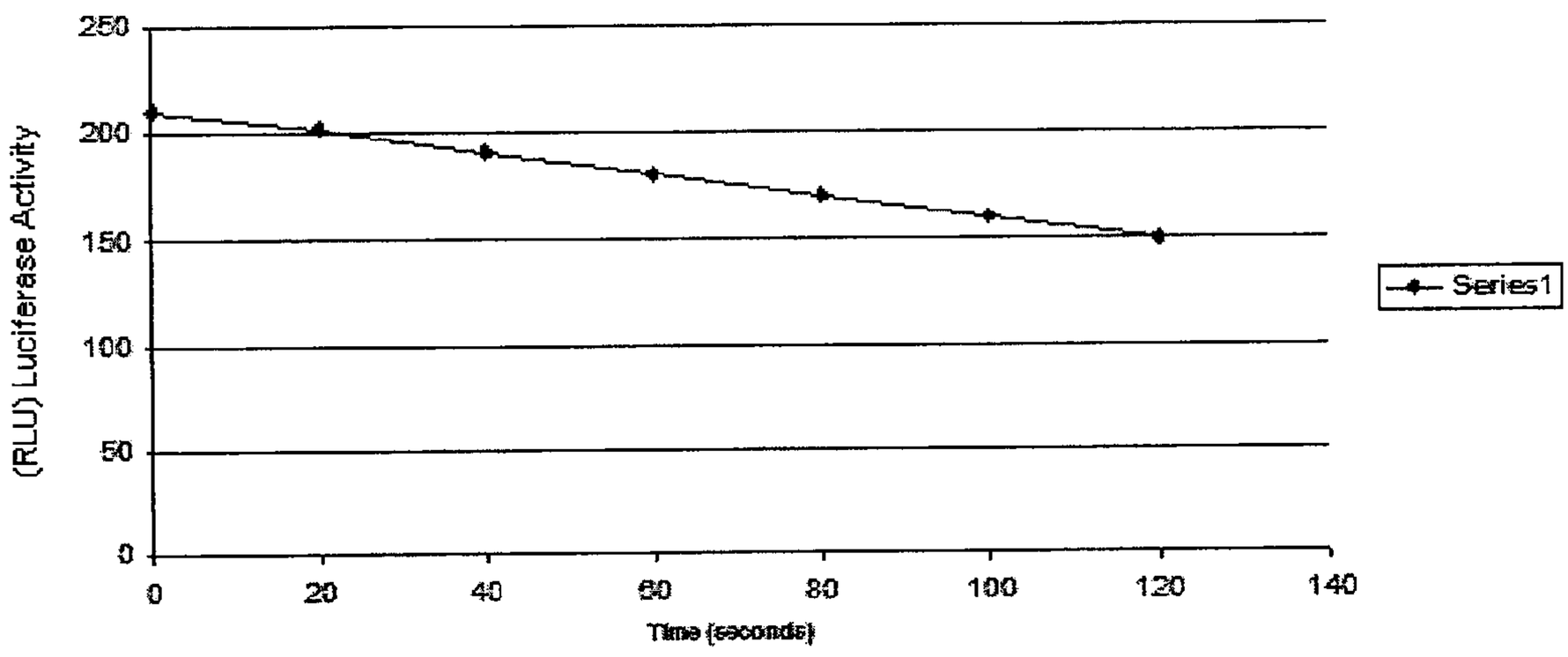
250
200
150
100
50
0
(RLU) Luciferase Activity
0
20
40
60
80
100
120
140
Time (seconds)
Series1

FIG. 18

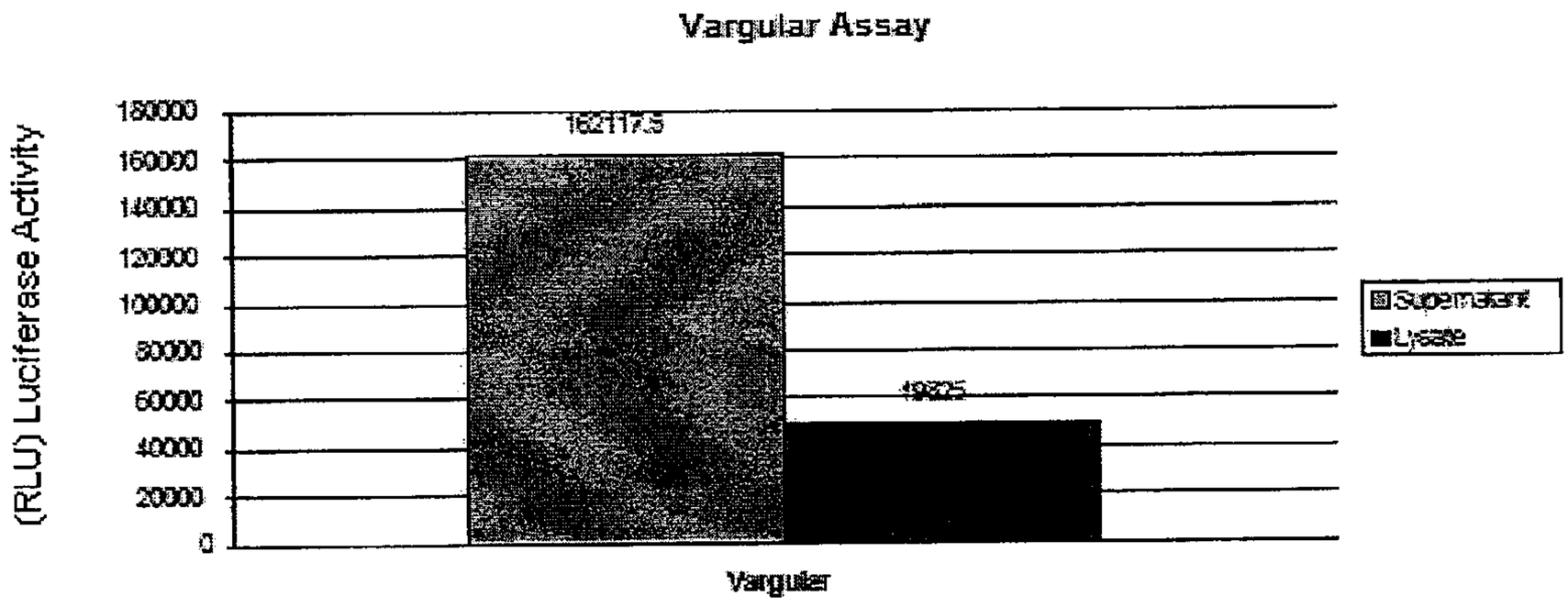
Vargular Assay
(RLU) Luciferase Activity
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
1621175
49225
Supernatant
Lysate
Vargular

FIG. 19

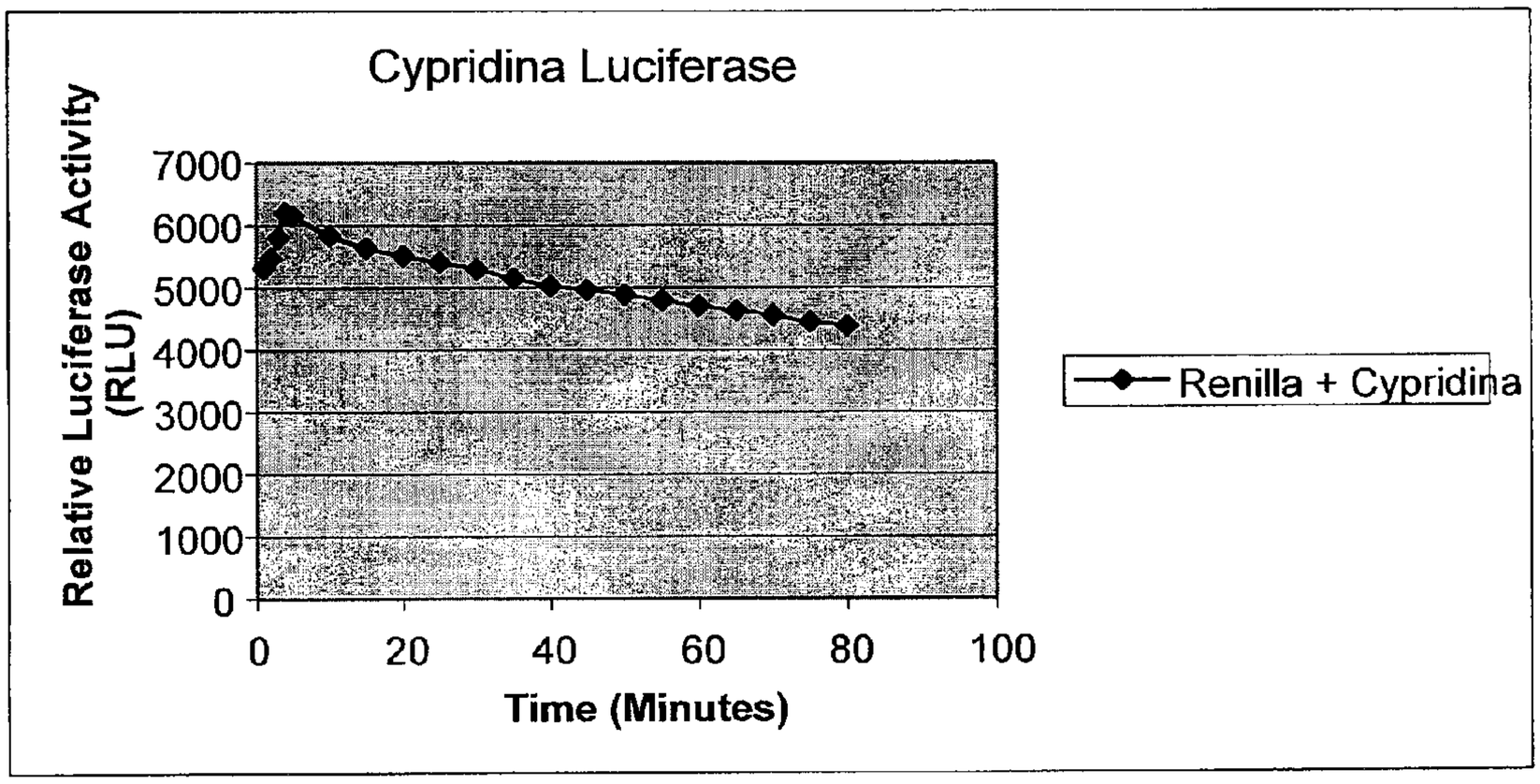
Cypridina Luciferase
Relative Luciferase Activity (RLU)
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Time (Minutes)
Renilla + Cypridina

FIG. 20A

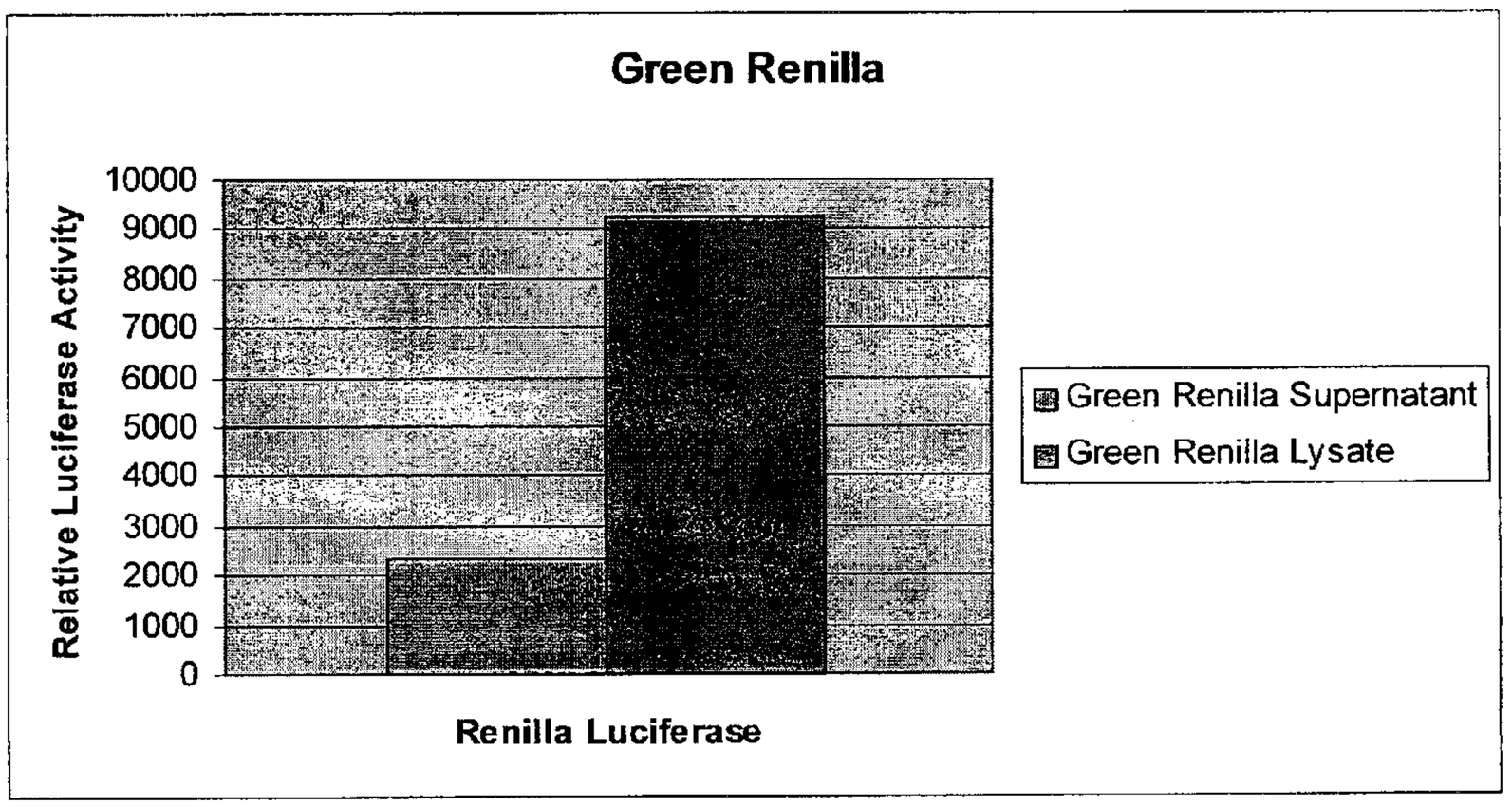

FIG. 20B

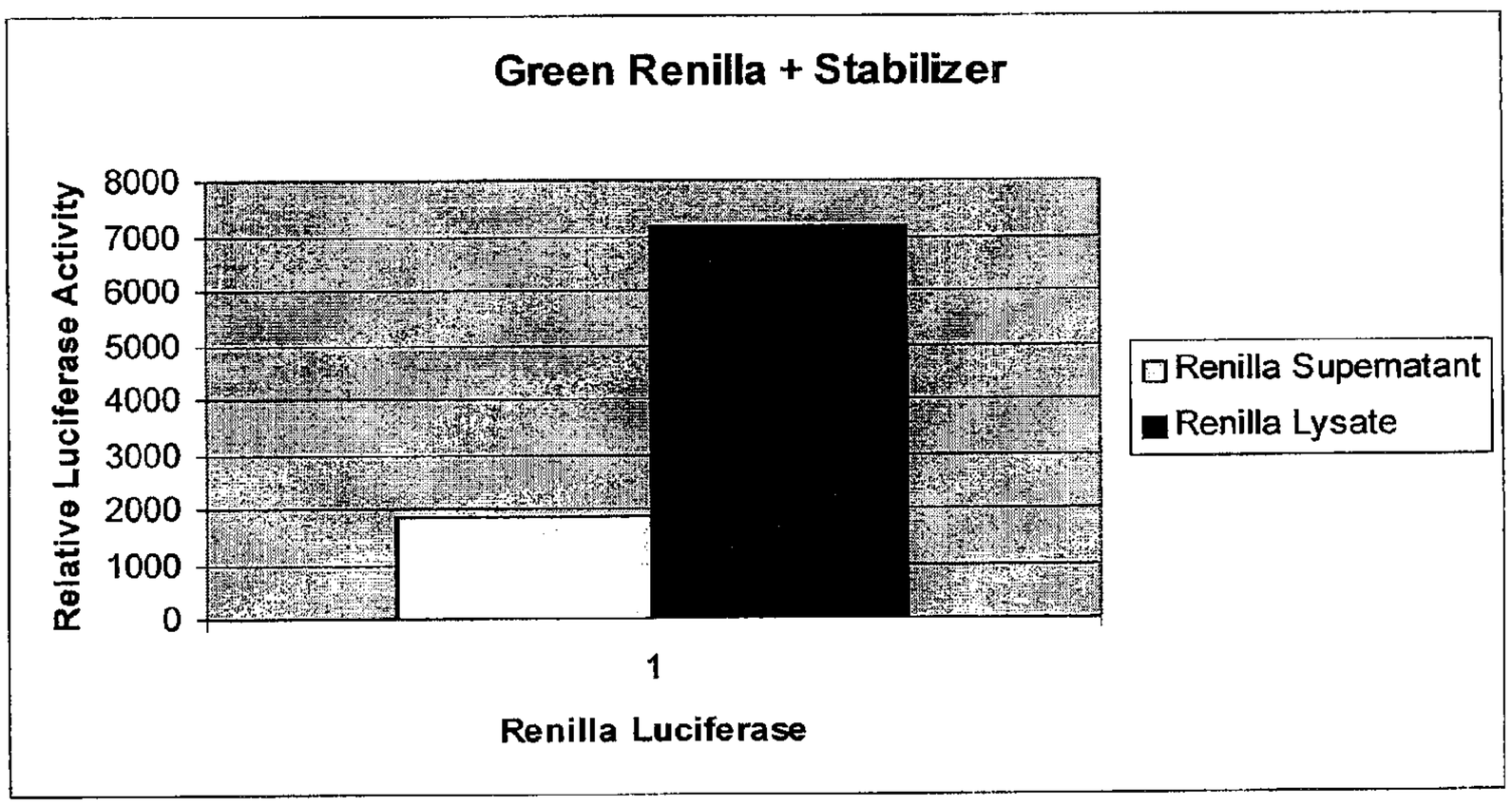

FIG. 21

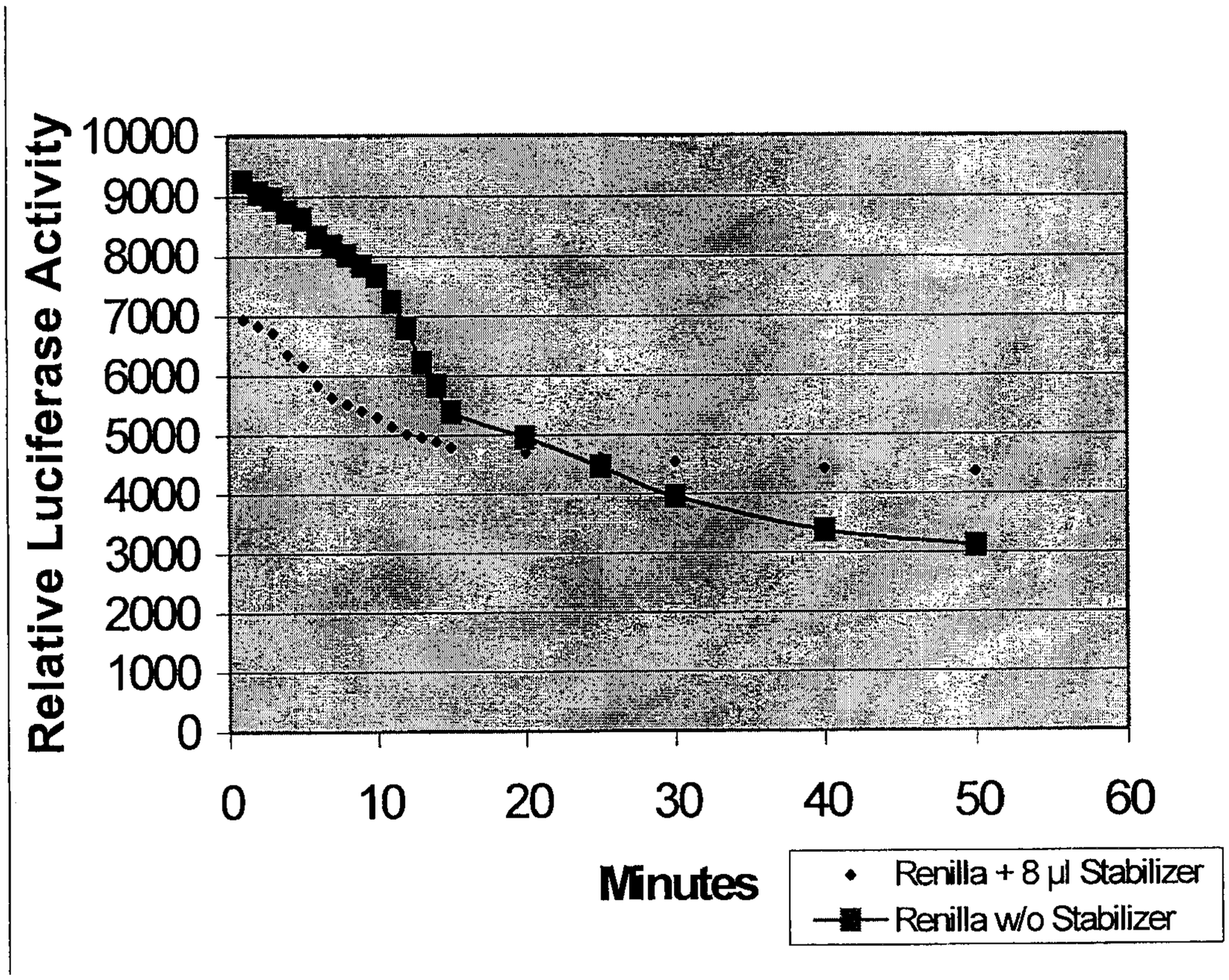
Relative Luciferase Activity
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
Minutes
Renilla + 8 µl Stabilizer
Renilla w/o Stabilizer

FIG. 22

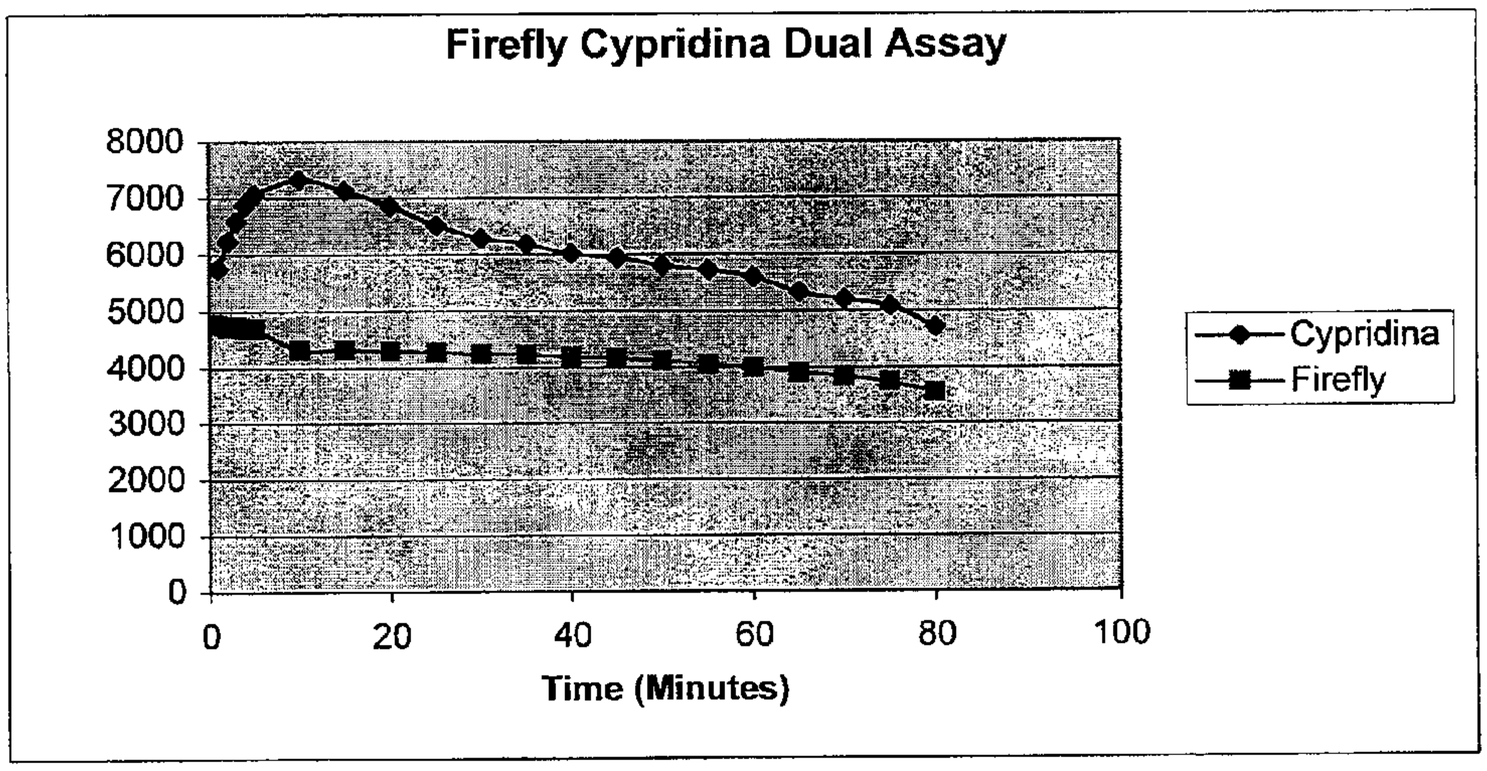
Firefly Cypridina Dual Assay
8000
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Time (Minutes)
Cypridina
Firefly

FIG. 23A

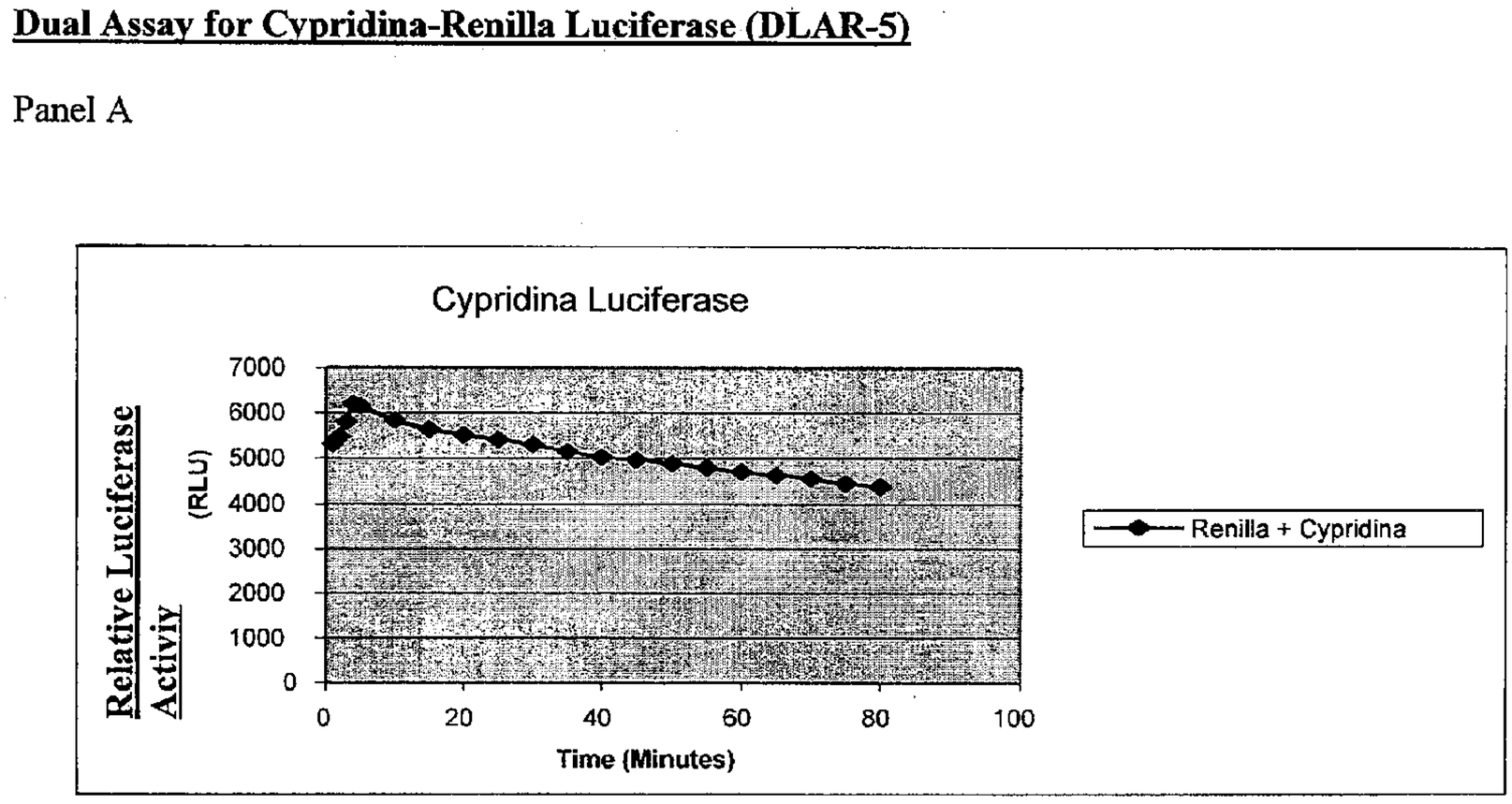

FIG. 23B

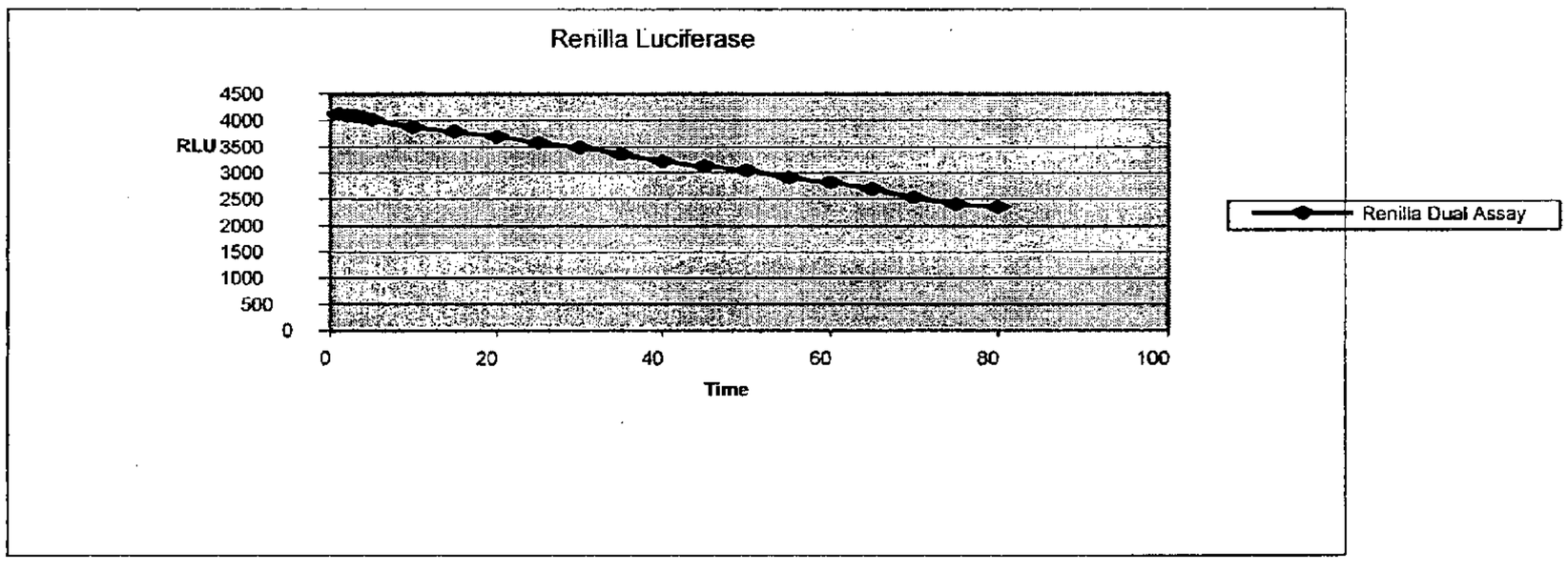

FIG. 24

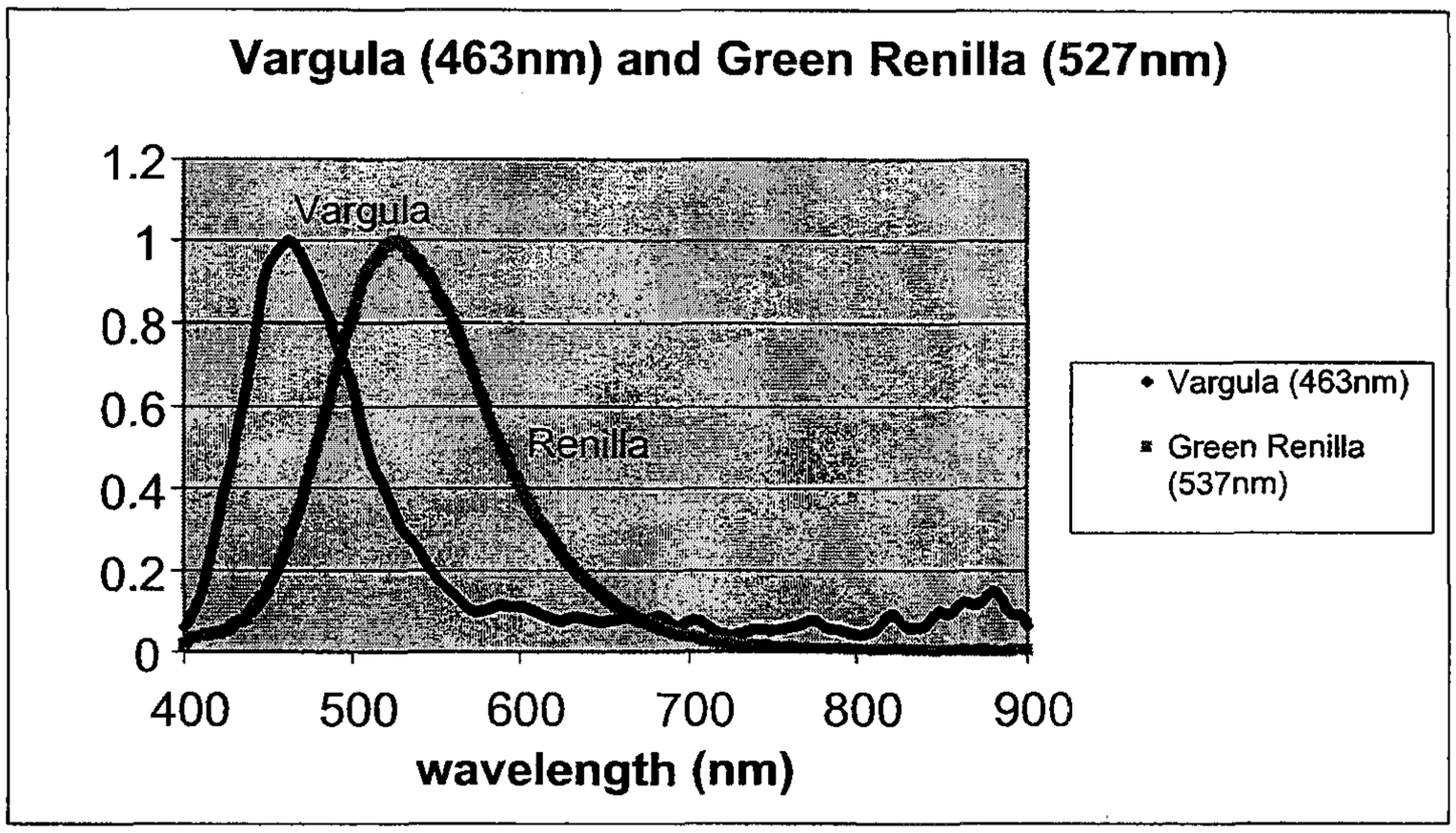
Vargula (463nm) and Green Renilla (527nm)
1.2
1
0.8
0.6
0.4
0.2
0
Vargula
Renilla
400
500
600
700
800
900
wavelength (nm)
Vargula (463nm)
Green Renilla (537nm)

FIG. 25

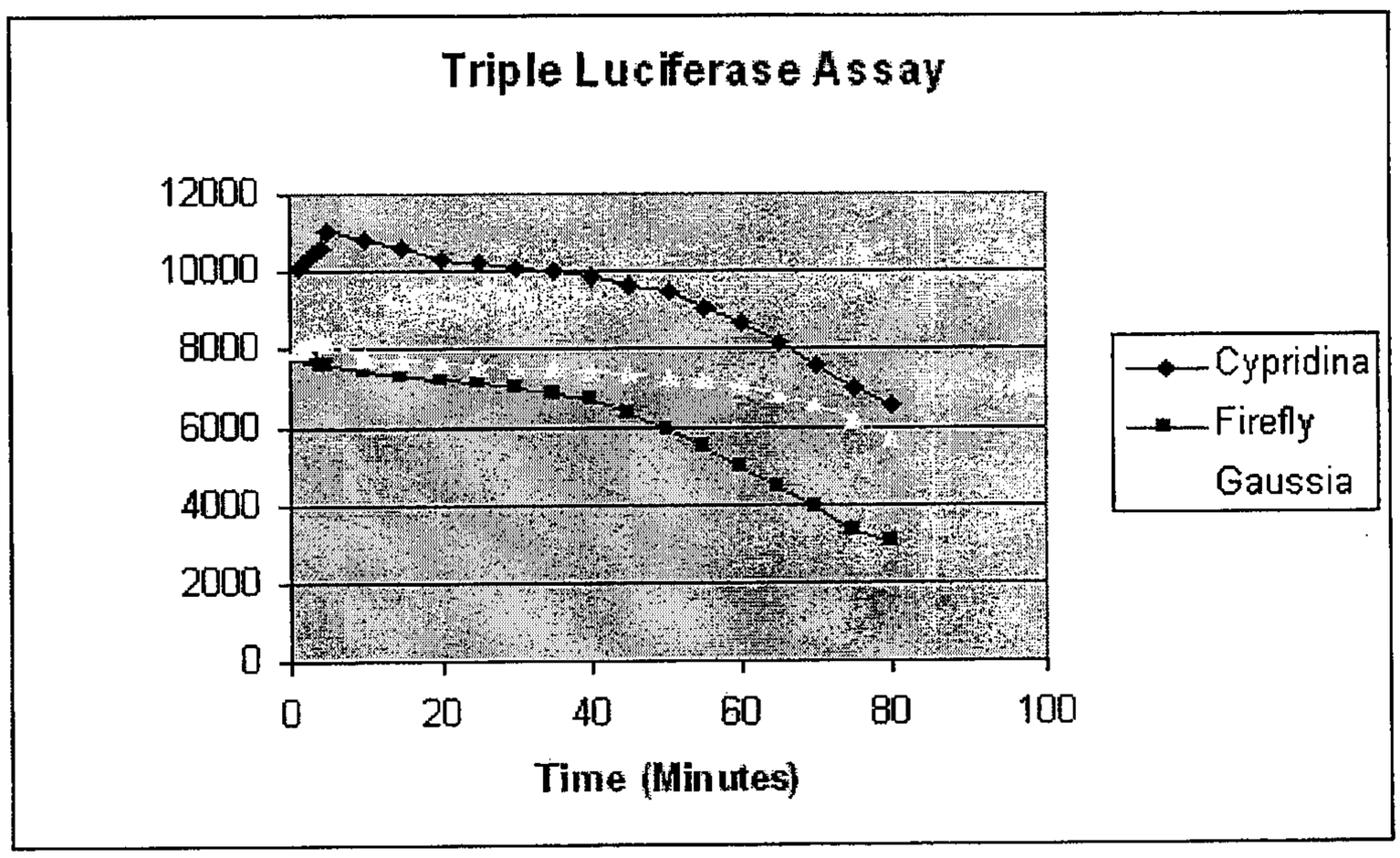
Triple Luciferase Assay
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
Time (Minutes)
Cypridina
Firefly
Gaussia

FIG. 26

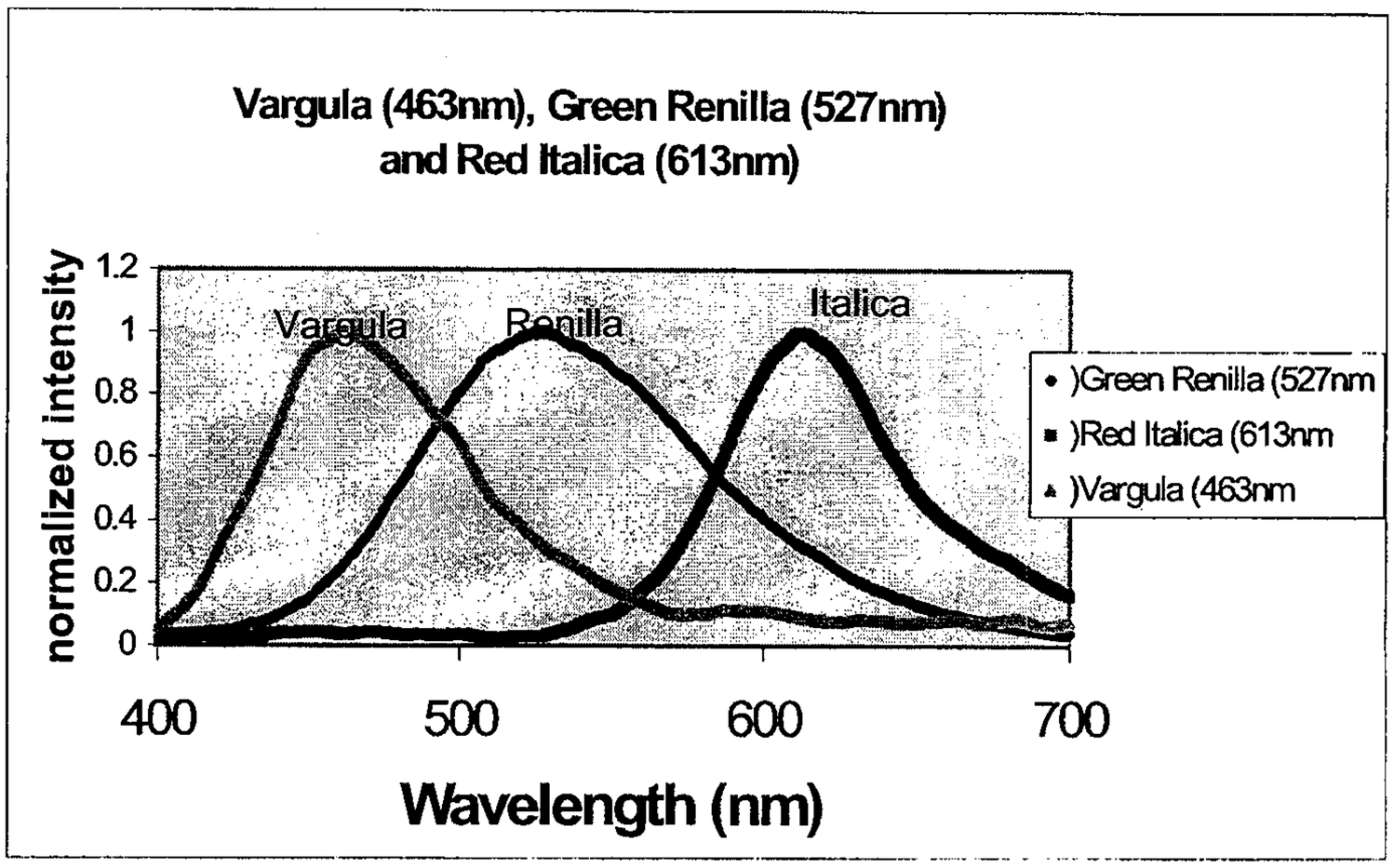
Vargula (463nm), Green Renilla (527nm)
and Red Italica (613nm)
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
Vargula
Renilla
Italica
400
500
600
700
Wavelength (nm)
)Green Renilla (527nm
)Red Italica (613nm
)Vargula (463nm

FIG. 27A

CMV Red Firefly Luciferase plasmid sequence:

ORIGIN

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc
901 gagctcggat ccagccacca tggaaacaga aagagaagaa aacgttgtct acggcccact
961 gccattctac ccgatcgagg agggctctgc cggcatccaa ttgcacaagt acatgcaaca
1021 atacgccaag ctcggcgcca tcgccttcag taacgccctg acaggcgtcg acatcagcta
1081 ccagcagtac ttcgacatca cgtgcagact cgccgaggct atgaagaact acggcatgaa
1141 gccagaagga cacatcgctc tctgtagcga gaactgcgaa gagttcttca ttcctgttct
1201 ggctggtctt tacatcggag ttacagtcgc gccaactaac gaaatttata cacttagaga
1261 gctgaaccac agtctgggga tagcccaacc tactatcgta ttctctagca ggaagggcct
1321 gcccaaagtg cttgaggtgc agaagaccgt gacttgcatc aaaaccattg tcatcctgga
1381 cagtaaggtc aacttcggcg gttatgactg cgtagagacc ttcattaaga aacacgtcga
1441 gctgggcttt cctgccacct catttgtgcc catcgacgtc aaagaccgga agcaccacat
1501 tgctctgctt atgaactctt ccggttccac agggctgccc aaaggagtag agatcactca
1561 cgaggccctg gtcacgagat tctctcacgc taaggaccct atatacggca atcaggtggc
1621 cccaggtacc gctatcctga ctgtcgtgcc tttccaccac ggcttcggaa tgttcactac
1681 tttgggctac tttgcctgcg gttaccggat tgtcatgctt actaagttcg acgaggagct
1741 tttcctgcgc acacttcagg attacaagtg cactacagta atcctggtgc cgacactgtt
1801 cgcaattctt aataggtctg agctccttga taagtttgac ctctctaacc tgactgaaat
1861 agccagcggt ggtgctccac ttgccaagga gatcggcgag gctgttgcaa gaagattcaa
1921 cctcccaggc gtccggcagg gatatggact caccgagact accagtgcct ttatcatcac
1981 tcctaagggc gacgacaagc cgggagccag cggcaaggtc gtgcctctgt tcaaggtgaa
2041 gattattgac ctcgatacca agaaaacgtt gggtgtcaac agacggggag aaatctgcgt
2101 gaaaggacca tctcttatgt tgggatacac gaacaatcct gaagccacca gagaaactat
2161 tgacgaggaa ggctggctgc acacgggtga catcgggtac tacgacgagg atgagcactt
2221 ctttatagtc gaccgcctga aatctctcat taagtataaa ggataccaag tgccaccagc
2281 tgaactggag tctgtgctcc tgcaacaccc taacattaga gatgctggtg tggccggggt
2341 tcccgacagc gaggcaggcg agctgcctgg agccgtcgtt gtgatggaaa agggaaagac
2401 aatgactgag aaagaaatcg tagactatgt aaactcccag gtggtcaacc acaagcggct
2461 gaggggcggc gtgcggttcg tagatgaagt ccccaagggg ctcacaggaa agatcgacgc
2521 gaaagttatc agggagatac tcaagaaacc tcaagcaggt gggtagtcta gaaataattc
2581 ttactgtcat gccaagtaag atgcttttct gtgctgcaat agcaggcatg ctggggatgc
2641 ggtgggctct atggcttctg aggcggaaag aaccagctgg ggctctaggg ggtatcccca
2701 cgcgccctgt agcggcgcat taagcgcggc gggtgtggtg gttacgcgca gcgtgaccgc
2761 tacacttgcc agcgccctag cgcccgctcc tttcgctttc ttcccttcct ttctcgccac
2821 gttcgccggc tttccccgtc aagctctaaa tcggggcatc cctttagggt tccgatttag
2881 tgctttacgg cacctcgacc ccaaaaaact tgattagggt gatggttcac gtagtgggcc
2941 atcgccctga tagacggttt ttcgcccttt gacgttggag tccacgttct ttaatagtgg
3001 actcttgttc caaactggaa caacactcaa ccctatctcg gtctattctt ttgatttata
3061 agggattttg gggatttcgg cctattggtt aaaaaatgag ctgatttaac aaaaatttaa

FIG. 27B

3121 cgcgaattaa ttctgtggaa tgtgtgtcag ttagggtgtg gaaagtcccc aggctcccca
3181 ggcaggcaga agtatgcaaa gcatgcatct caattagtca gcaaccaggt gtggaaagtc
3241 cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt cagcaaccat
3301 agtcccgccc ctaactccgc ccatcccgcc cctaactccg cccagttccg cccattctcc
3361 gccccatggc tgactaattt tttttattta tgcagaggcc gaggccgcct ctgcctctga
3421 gctattccag aagtagtgag gaggcttttt tggaggccta ggcttttgca aaaagctccc
3481 gggagcttgt atatccattt tcggatctga tcaagagaca ggatgaggat cgtttcgcat
3541 gattgaacaa gatggattgc acgcaggttc tccggccgct tgggtggaga ggctattcgg
3601 ctatgactgg gcacaacaga caatcggctg ctctgatgcc gccgtgttcc ggctgtcagc
3661 gcaggggcgc ccggttcttt ttgtcaagac cgacctgtcc ggtgccctga atgaactgca
3721 ggacgaggca gcgcggctat cgtggctggc cacgacgggc gttccttgcg cagctgtgct
3781 cgacgttgtc actgaagcgg gaagggactg gctgctattg ggcgaagtgc cggggcagga
3841 tctcctgtca tctcaccttg ctcctgccga gaaagtatcc atcatggctg atgcaatgcg
3901 gcggctgcat acgcttgatc cggctacctg cccattcgac caccaagcga aacatcgcat
3961 cgagcgagca cgtactcgga tggaagccgg tcttgtcgat caggatgatc tggacgaaga
4021 gcatcagggg ctcgcgccag ccgaactgtt cgccaggctc aaggcgcgca tgcccgacgg
4081 cgaggatctc gtcgtgaccc atggcgatgc ctgcttgccg aatatcatgg tggaaaatgg
4141 ccgcttttct ggattcatcg actgtggccg gctgggtgtg gcggaccgct atcaggacat
4201 agcgttggct acccgtgata ttgctgaaga gcttggcggc gaatgggctg accgcttcct
4261 cgtgctttac ggtatcgccg ctcccgattc gcagcgcatc gccttctatc gccttcttga
4321 cgagttcttc tgagcgggac tctggggttc gaaatgaccg accaagcgac gcccaacctg
4381 ccatcacgag atttcgattc caccgccgcc ttctatgaaa ggttgggctt cggaatcgtt
4441 ttccgggacg ccggctggat gatcctccag cgcggggatc tcatgctgga gttcttcgcc
4501 cacccccaact tgtttattgc agcttataat ggttacaaat aaagcaatag catcacaaat
4561 ttcacaaata aagcattttt ttcactgcat tctagttgtg gtttgtccaa actcatcaat
4621 gtatcttatc atgtctgtat accgtcgacc tctagctaga gcttggcgta atcatggtca
4681 tagctgtttc ctgtgtgaaa ttgttatccg ctcacaattc cacacaacat acgagccgga
4741 agcataaagt gtaaagcctg gggtgcctaa tgagtgagct aactcacatt aattgcgttg
4801 cgctcactgc ccgctttcca gtcgggaaac ctgtcgtgcc agctgcatta atgaatcggc
4861 caacgcgcgg ggagaggcgg tttgcgtatt gggcgctctt ccgcttcctc gctcactgac
4921 tcgctgcgct cggtcgttcg gctgcggcga gcggtatcag ctcactcaaa ggcggtaata
4981 cggttatcca cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa
5041 aaggccagga accgtaaaaa ggccgcgttg ctggcgtttt tccataggct ccgcccccct
5101 gacgagcatc acaaaaatcg acgctcaagt cagaggtggc gaaacccgac aggactataa
5161 agataccagg cgtttccccc tggaagctcc ctcgtgcgct ctcctgttcc gaccctgccg
5221 cttaccggat acctgtccgc ctttctccct tcgggaagcg tggcgctttc tcaatgctca
5281 cgctgtaggt atctcagttc ggtgtaggtc gttcgctcca agctgggctg tgtgcacgaa
5341 ccccccgttc agcccgaccg ctgcgcctta tccggtaact atcgtcttga gtccaacccg
5401 gtaagacacg acttatcgcc actggcagca gccactggta acaggattag cagagcgagg
5461 tatgtaggcg gtgctacaga gttcttgaag tggtggccta actacggcta cactagaagg
5521 acagtatttg gtatctgcgc tctgctgaag ccagttacct tcggaaaaag agttggtagc
5581 tcttgatccg gcaaacaaac caccgctggt agcggtggtt tttttgtttg caagcagcag
5641 attacgcgca gaaaaaaagg atctcaagaa gatcctttga tcttttctac ggggtctgac
5701 gctcagtgga acgaaaactc acgttaaggg attttggtca tgagattatc aaaaaggatc
5761 ttcacctaga tcctttttaaa ttaaaaatga agttttaaat caatctaaag tatatatgag
5821 taaacttggt ctgacagtta ccaatgctta atcagtgagg cacctatctc agcgatctgt
5881 ctatttcgtt catccatagt tgcctgactc cccgtcgtgt agataactac gatacgggag
5941 ggcttaccat ctggccccag tgctgcaatg ataccgcgag acccacgctc accggctcca
6001 gatttatcag caataaacca gccagccgga agggccgagc gcagaagtgg tcctgcaact
6061 ttatccgcct ccatccagtc tattaattgt tgccgggaag ctagagtaag tagttcgcca
6121 gttaatagtt tgcgcaacgt tgttgccatt gctacaggca tcgtggtgtc acgctcgtcg
6181 tttggtatgg cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatccccc
6241 atgttgtgca aaaaagcggt tagctccttc ggtcctccga tcgttgtcag aagtaagttg
6301 gccgcagtgt tatcactcat ggttatggca gcactgcata attctcttac tgtcatgcca

FIG. 27C

6361 tccgtaagat gcttttctgt gactggtgag tactcaacca agtcattctg agaatagtgt
6421 atgcggcgac cgagttgctc ttgcccggcg tcaatacggg ataataccgc gccacatagc
6481 agaactttaa aagtgctcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc
6541 ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg cacccaactg atcttcagca
6601 tcttttactt tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa
6661 aagggaataa gggcgacacg gaaatgttga atactcatac tcttcctttt tcaatattat
6721 tgaagcattt atcagggtta ttgtctcatg agcggataca tatttgaatg tatttagaaa
6781 aataaacaaa taggggttcc gcgcacattt ccccgaaaag tgccacctga cgtc

FIG. 28

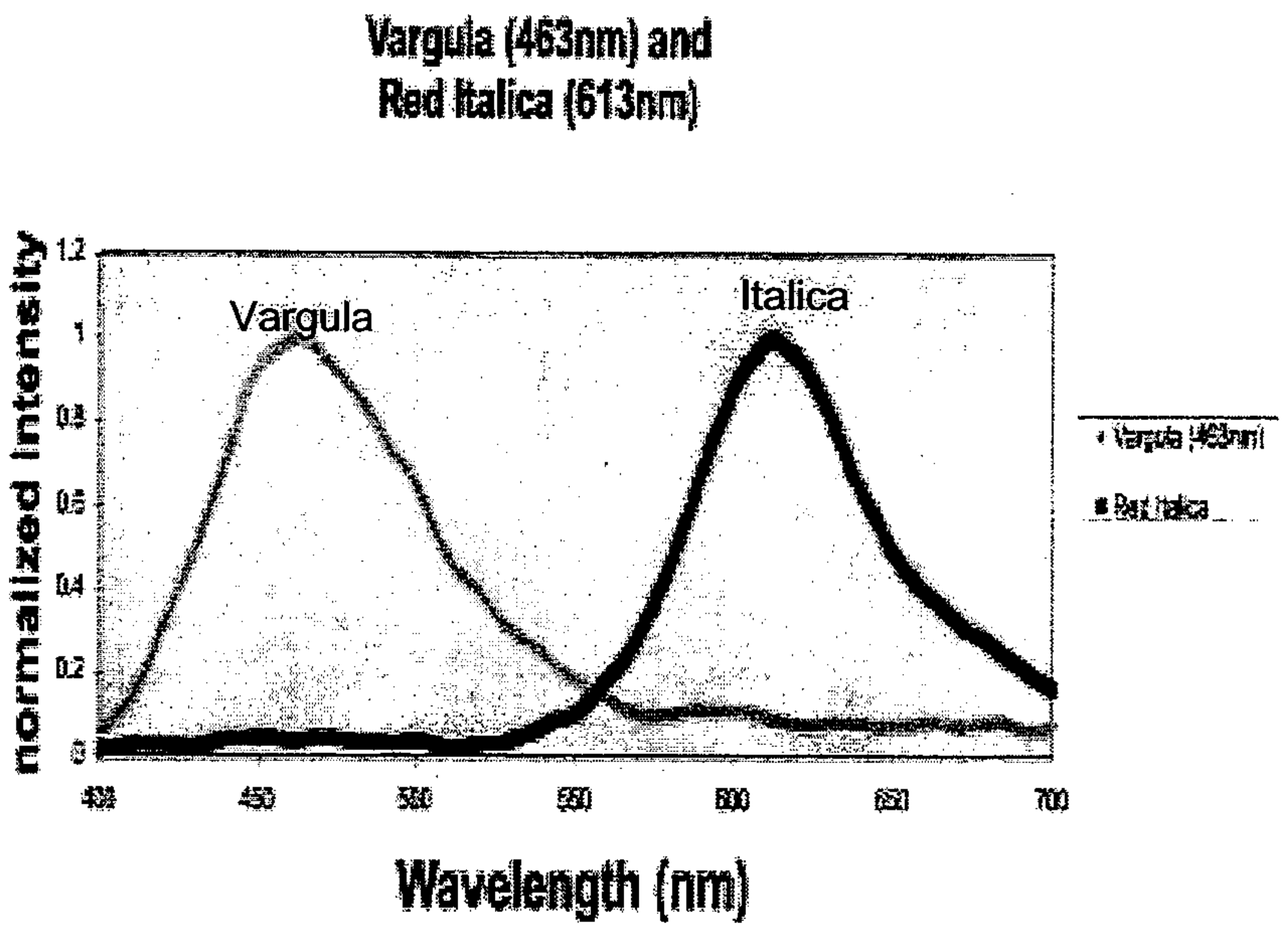
Varguia (463nm) and
Red Italica (613nm)
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
Vargula
Italica
450
550
650
700
Wavelength (nm)

FIG. 29B

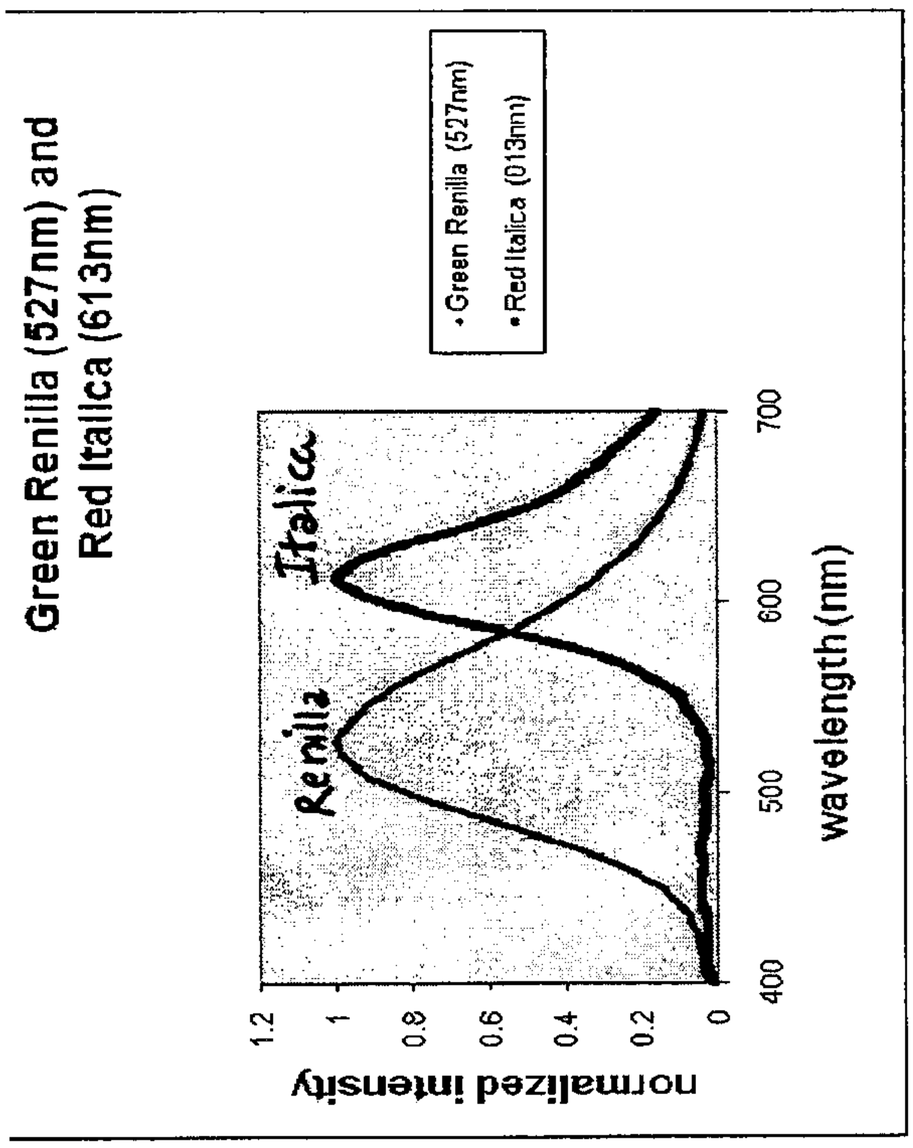

FIG. 29A

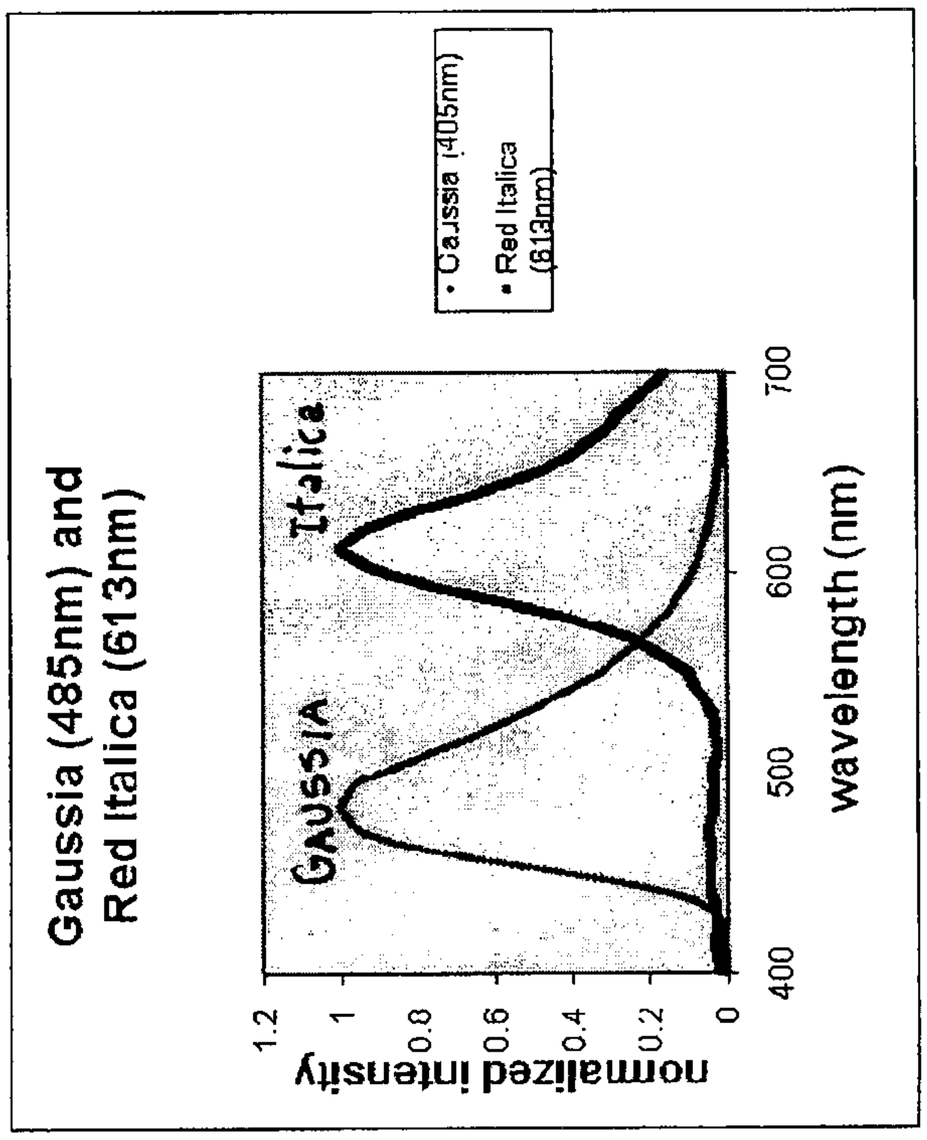

FIG. 30A

A mammalian expression vector expressing Red emitting firefly luciferase (human codon optimized signal) under control of the CMV promoter) (SEQ ID NO: 3)

Size: 6827 bases

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgaccttа tgggactttc ctacttggca gtacatctac gtattagtca
601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc
901 gagctcggat ccatggaaac agaaagagaa gaaaacgttg tctacggccc actgccattc
961 tacccgatcg aggagggctc tgccggcatc caattgcaca agtacatgca acaatacgcc
1021 aagctcggcg ccatcgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081 tacttcgaca tcacgtgcag actcgccgag gctatgaaga actacggcat gaagccagaa
1141 ggacacatcg ctctctgtag cgagaactgc gaagagttct tcattcctgt tctggctggt
1201 ctttacatcg gagttacagt cgcgccaact aacgaaattt atacacttag agagctgaac
1261 cacagtctgg ggatagccca acctactatc gtattctcta gcaggaaggg cctgcccaaa
1321 gtgcttgagg tgcagaagac cgtgacttgc atcaaaacca ttgtcatcct ggacagtaag
1381 gtcaacttcg gcggttatga ctgcgtagag accttcatta agaaacacgt cgagctgggc
1441 tttcctgcca cctcatttgt gcccatcgac gtcaaagacc ggaagcacca cattgctctg
1501 cttatgaact cttccggttc cacagggctg cccaaaggag tagagatcac tcacgaggcc
1561 ctggtcacga gattctctca cgctaaggac cctatatacg gcaatcaggt ggccccaggt
1621 accgctatcc tgactgtcgt gcctttccac cacggcttcg gaatgttcac tactttgggc
1681 tactttgcct gcggttaccg gattgtcatg cttactaagt tcgacgagga gcttttcctg
1741 cgcacacttc aggattacaa gtgcactaca gtaatcctgg tgccgacact gttcgcaatt
1801 cttaataggt ctgagctcct tgataagttt gacctctcta acctgactga aatagccagc
1861 ggtggtgctc cacttgccaa ggagatcggc gaggctgttg caagaagatt caacctccca
1921 ggcgtccggc agggatatgg actcaccgag actaccagtg cctttatcat cactcctaag
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgttcaaggt gaagattatt
2041 gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101 ccatctctta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca cttctttata
2221 gtcgaccgcc tgaaatctct cattaagtat aaaggatacc aagtgccacc agctgaactg
2281 gagtctgtgc tcctgcaaca ccctaacatt agagatgctg gtgtggccgg ggttcccgac
2341 agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401 gagaaagaaa tcgtagacta tgtaaactcc caggtggtca accacaagcg gctgaggggc
2461 ggcgtgcggt tcgtagatga agtccccaag gggctcacag gaaagatcga cgcgaaagtt
2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctagatctag aaataattct
2581 tactgtcatg ccaagtaaga tgcttttctg tgctgcaata gcaggcatgc tggggatgcg
2641 gtgggctcta tggcttctga ggcggaaaga accagctggg gctctagggg gtatccccac
2701 gcgccctgta gcggcgcatt aagcgcggcg ggtgtggtgg ttacgcgcag cgtgaccgct
2761 acacttgcca gcgccctagc gcccgctcct ttcgctttct tcccttcctt tctcgccacg
2821 ttcgccggct ttccccgtca agctctaaat cggggcatcc ctttagggtt ccgatttagt
2881 gctttacggc acctcgaccc caaaaaactt gattagggtg atggttcacg tagtgggcca
2941 tcgccctgat agacggtttt tcgccctttg acgttggagt ccacgttctt taatagtgga
3001 ctcttgttcc aaactggaac aacactcaac cctatctcgg tctattcttt tgatttataa
3061 gggattttgg ggatttcggc ctattggtta aaaaatgagc tgatttaaca aaaatttaac
3121 gcgaattaat tctgtggaat gtgtgtcagt tagggtgtgg aaagtcccca ggctccccag
3181 gcaggcagaa gtatgcaaag catgcatctc aattagtcag caaccaggtg tggaaagtcc
3241 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata
3301 gtcccgcccc taactccgcc catcccgccc ctaactccgc ccagttccgc ccattctccg

FIG. 30B

3361 ccccatggct gactaatttt ttttatttat gcagaggccg aggccgcctc tgcctctgag
3421 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttgcaa aaagctcccg
3481 ggagcttgta tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg
3541 attgaacaag atggattgca cgcaggttct ccggccgctt gggtggagag gctattcggc
3601 tatgactggg cacaacagac aatcggctgc tctgatgccg ccgtgttccg gctgtcagcg
3661 caggggcgcc cggttctttt tgtcaagacc gacctgtccg gtgccctgaa tgaactgcag
3721 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc
3781 gacgttgtca ctgaagcggg aagggactgg ctgctattgg gcgaagtgcc ggggcaggat
3841 ctcctgtcat ctcaccttgc tcctgccgag aaagtatcca tcatggctga tgcaatgcgg
3901 cggctgcata cgcttgatcc ggctacctgc ccattcgacc accaagcgaa acatcgcatc
3961 gagcgagcac gtactcggat ggaagccggt cttgtcgatc aggatgatct ggacgaagag
4021 catcaggggc tcgcgccagc cgaactgttc gccaggctca aggcgcgcat gcccgacggc
4081 gaggatctcg tcgtgaccca tggcgatgcc tgcttgccga atatcatggt ggaaaatggc
4141 cgcttttctg gattcatcga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata
4201 gcgttggcta cccgtgatat tgctgaagag cttggcggcg aatgggctga ccgcttcctc
4261 gtgctttacg gtatcgccgc tcccgattcg cagcgcatcg ccttctatcg ccttcttgac
4321 gagttcttct gagcgggact ctggggttcg aaatgaccga ccaagcgacg cccaacctgc
4381 catcacgaga tttcgattcc accgccgcct tctatgaaag gttgggcttc ggaatcgttt
4441 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttcttcgccc
4501 accccaactt gtttattgca gcttataatg gttacaaata aagcaatagc atcacaaatt
4561 tcacaaataa agcatttttt tcactgcatt ctagttgtgg tttgtccaaa ctcatcaatg
4621 tatcttatca tgtctgtata ccgtcgacct ctagctagag cttggcgtaa tcatggtcat
4681 agctgtttcc tgtgtgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa
4741 gcataaagtg taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc
4801 gctcactgcc cgctttccag tcgggaaacc tgtcgtgcca gctgcattaa tgaatcggcc
4861 aacgcgcggg gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg ctcactgact
4921 cgctgcgctc ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag gcggtaatac
4981 ggttatccac agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa
5041 aggccaggaa ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgcccccctg
5101 acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa
5161 gataccaggc gtttcccccc ggaagctccc tcgtgcgctc tcctgttccg accctgccgc
5221 ttaccggata cctgtccgcc tttctccctt cgggaagcgt ggcgctttct caatgctcac
5281 gctgtaggta tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac
5341 ccccccgttca gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg
5401 taagacacga cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt
5461 atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagga
5521 cagtatttgg tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct
5581 cttgatccgg caaacaaacc accgctggta gcggtggttt ttttgtttgc aagcagcaga
5641 ttacgcgcag aaaaaaagga tctcaagaag atcctttgat cttttctacg gggtctgacg
5701 ctcagtggaa cgaaaactca cgttaaggga ttttggtcat gagattatca aaaaggatct
5761 tcacctagat ccttttaaat taaaaatgaa gttttaaatc aatctaaagt atatatgagt
5821 aaacttggtc tgacagttac caatgcttaa tcagtgaggc acctatctca gcgatctgtc
5881 tatttcgttc atccatagtt gcctgactcc ccgtcgtgta gataactacg atacgggagg
5941 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag
6001 atttatcagc aataaaccag ccagccggaa gggccgagcg cagaagtggt cctgcaactt
6061 tatccgcctc catccagtct attaattgtt gccgggaagc tagagtaagt agttcgccag
6121 ttaatagttt gcgcaacgtt gttgccattg ctacaggcat cgtggtgtca cgctcgtcgt
6181 ttggtatggc ttcattcagc tccggttccc aacgatcaag gcgagttaca tgatcccccа
6241 tgttgtgcaa aaaagcggtt agctccttcg gtcctccgat cgttgtcaga agtaagttgg
6301 ccgcagtgtt atcactcatg gttatggcag cactgcataa ttctcttact gtcatgccat
6361 ccgtaagatg cttttctgtg actggtgagt actcaaccaa gtcattctga gaatagtgta
6421 tgcggcgacc gagttgctct tgcccggcgt caatacggga taataccgcg ccacatagca
6481 gaactttaaa agtgctcatc attggaaaac gttcttcggg gcgaaaactc tcaaggatct
6541 taccgctgtt gagatccagt tcgatgtaac ccactcgtgc acccaactga tcttcagcat
6601 cttttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa
6661 agggaataag ggcgacacgg aaatgttgaa tactcatact cttccttttt caatattatt
6721 gaagcattta tcagggttat tgtctcatga gcggatacat atttgaatgt atttagaaaa
6781 ataaacaaat aggggttccg cgcacatttc cccgaaaagt gccacctgac gtc

FIG. 31A

Sequence and Features of pCMV GrFLUC Vector:
A mammalian expression vector expressing human codon optimized green firefly luciferase (Luciola Italica) under control of the CMV promoter (SEQ ID NO: 4)
Size: 6827 bases

pCMV-GrFLuc (6827 bp)
CMV promoter bases: 209-863
Green emitting firefly luciferase gene: 907-2560
T7 promoter bases: 1827-1845
Polylinker bases: 1852-1870
SP6 promoter: 2576-2593
Synthetic polyadenylation site: 2560-2604
SV40 promoter bases: 3145-3480
SV40 origin of replication: bases: 3259-3344
Neomycin ORF : bases 3516- 4310
SV40 PolyA: bases 4365-4737
ColE1 origin: bases 3934-4607
Ampicillin ORF: bases 4752-5612

```
   1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
  61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
 121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
 181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
 241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
 301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
 361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
 421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
 481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
 541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
 601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
 661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
 721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
 781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
 841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc
 901 gagctcggat ccatggaaac agaaagagaa gaaaacgttg tctacggccc actgccattc
 961 tacccgatcg aggagggctc tgccggcatc caattgcaca agtacatgca acaatacgcc
1021 aagctcggcg ccatcgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081 tacttcgaca tcacgtgcag actcgccgag gctatgaaga actacggcat gaagccagaa
1141 ggacacatcg ctctctgtag cgagaactgc gaagagttct tcattcctgt tctggctggt
1201 ctttacatcg gagttacagt cgcgccaact aacgaaattt atacacttag agagctgaac
1261 cacagtctgg ggatagccca acctactatc gtattctcta gcaggaaggg cctgcccaaa
1321 gtgcttgagg tgcagaagac cgtgacttgc atcaaaacca ttgtcatcct ggacagtaag
1381 gtcaacttcg gcggttatga ctgcgtagag accttcatta agaaacacgt cgagctgggc
1441 tttcctgcca cctcatttgt gcccatcgac gtcaaagacc ggaagcacca cattgctctg
1501 cttatgaact cttccggttc cacagggctg cccaaaggag tagagatcac tcacgaggcc
1561 ctggtcacga gattctctca cgctaaggac cctatatacg gcaatcaggt ggccccaggt
1621 accgctatcc tgactgtcat ccctttccac cacgccttcg gaatgagcac tactttgggc
1681 tactttgcct gcggttaccg gattgtcatg cttactaagt tcgacgagga gcttttcctg
1741 cgcacacttc aggattacaa gtgcactagc gtaatcctgg tgccgacact gttcgcaatt
1801 cttaataggt ctgagctcct tgataagttt gacctctcta acctgactga aatagccagc
1861 ggtggtgctc cacttgccaa ggagatcggc gaggctgttg caagaagatt caacctccca
1921 ggcgtccggc agggatatgg actcaccgag actaccagtg cctttatcat cactcctaag
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgttcaaggt gaagattatt
2041 gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101 ccatctctta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca cttctttata
2221 gtcgaccgcc tgaaatctct cattaagtat aaaggatacc aagtgccacc agctgaactg
2281 gagtctgtgc tcctgcaaca ccctaacatt agagatgctg gtgtggccgg ggttcccgac
2341 agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401 gagaaagaaa tcgtagacta tgtaaactcc caggtggtca accacaagcg gctgaggggc
2461 ggcgtgcggt tcgtagatga agtccccaag gggctcacag gaaagatcga cgcgaaagtt
```

FIG. 31B

2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctagaaataa ttcttactgt
2581 catgccaagt aagatgcttt tctgtgctgc aatagcaggc atgctgggga tgcggtgggc
2641 tctatggctt ctgaggcgga aagaaccagc tggggctcta gggggtatcc ccacgcgccc
2701 tgtagcggcg cattaagcgc ggcgggtgtg gtggttacgc gcagcgtgac cgctacactt
2761 gccagcgccc tagcgcccgc tcctttcgct ttcttccctt cctttctcgc cacgttcgcc
2821 ggctttcccc gtcaagctct aaatcggggc atcccttag ggttccgatt tagtgcttta
2881 cggcacctcg accccaaaaa acttgattag ggtgatggtt cacgtagtgg gccatcgccc
2941 tgatagacgg tttttcgccc tttgacgttg gagtccacgt tctttaatag tggactcttg
3001 ttccaaactg gaacaacact caaccctatc tcggtctatt cttttgattt ataagggatt
3061 ttggggattt cggcctattg gttaaaaaat gagctgattt aacaaaaatt taacgcgaat
3121 taattctgtg gaatgtgtgt cagttagggt gtggaaagtc cccaggctcc ccaggcaggc
3181 agaagtatgc aaagcatgca tctcaattag tcagcaacca ggtgtggaaa gtccccaggc
3241 tccccagcag gcagaagtat gcaaagcatg catctcaatt agtcagcaac catagtcccg
3301 cccctaactc cgcccatccc gcccctaact ccgcccagtt ccgcccattc tccgccccat
3361 ggctgactaa ttttttttat ttatgcagag gccgaggccg cctctgcctc tgagctattc
3421 cagaagtagt gaggaggctt ttttggaggc ctaggctttt gcaaaaagct cccgggagct
3481 tgtatatcca ttttcggatc tgatcaagag acaggatgag gatcgtttcg catgattgaa
3541 caagatggat tgcacgcagg ttctccggcc gcttgggtgg agaggctatt cggctatgac
3601 tgggcacaac agacaatcgg ctgctctgat gccgccgtgt tccggctgtc agcgcagggg
3661 cgcccggttc tttttgtcaa gaccgacctg tccggtgccc tgaatgaact gcaggacgag
3721 gcagcgcggc tatcgtggct ggccacgacg ggcgttcctt gcgcagctgt gctcgacgtt
3781 gtcactgaag cgggaaggga ctggctgcta ttgggcgaag tgccggggca ggatctcctg
3841 tcatctcacc ttgctcctgc cgagaaagta tccatcatgg ctgatgcaat gcggcggctg
3901 catacgcttg atccggctac ctgcccattc gaccaccaag cgaaacatcg catcgagcga
3961 gcacgtactc ggatggaagc cggtcttgtc gatcaggatg atctggacga agagcatcag
4021 gggctcgcgc cagccgaact gttcgccagg ctcaaggcgc gcatgcccga cggcgaggat
4081 ctcgtcgtga cccatggcga tgcctgcttg ccgaatatca tggtggaaaa tggccgcttt
4141 tctggattca tcgactgtgg ccggctgggt gtggcggacc gctatcagga catagcgttg
4201 gctacccgtg atattgctga agagcttggc ggcgaatggg ctgaccgctt cctcgtgctt
4261 tacggtatcg ccgctcccga ttcgcagcgc atcgccttct atcgccttct tgacgagttc
4321 ttctgagcgg gactctgggg ttcgaaatga ccgaccaagc gacgcccaac ctgccatcac
4381 gagatttcga ttccaccgcc gccttctatg aaaggttggg cttcggaatc gttttccggg
4441 acgccggctg gatgatcctc cagcgcgggg atctcatgct ggagttcttc gcccacccca
4501 acttgtttat tgcagcttat aatggttaca ataaagcaa tagcatcaca aatttcacaa
4561 ataaagcatt tttttcactg cattctagtt gtggtttgtc caaactcatc aatgtatctt
4621 atcatgtctg tataccgtcg acctctagct agagcttggc gtaatcatgg tcatagctgt
4681 ttcctgtgtg aaattgttat ccgctcacaa ttccacacaa catacgagcc ggaagcataa
4741 agtgtaaagc ctggggtgcc taatgagtga gctaactcac attaattgcg ttgcgctcac
4801 tgcccgcttt ccagtcggga aacctgtcgt gccagctgca ttaatgaatc ggccaacgcg
4861 cggggagagg cggtttgcgt attgggcgct cttccgcttc ctcgctcact gactcgctgc
4921 gctcggtcgt tcggctgcgg cgagcggtat cagctcactc aaaggcggta atacggttat
4981 ccacagaatc aggggataac gcaggaaaga acatgtgagc aaaaggccag caaaaggcca
5041 ggaaccgtaa aaaggccgcg ttgctggcgt ttttccatag gctccgcccc cctgacgagc
5101 atcacaaaaa tcgacgctca agtcagaggt ggcgaaaccc gacaggacta taaagatacc
5161 aggcgtttcc ccctggaagc tccctcgtgc gctctcctgt tccgaccctg ccgcttaccg
5221 gatacctgtc cgcctttctc ccttcgggaa gcgtggcgct ttctcaatgc tcacgctgta
5281 ggtatctcag ttcggtgtag gtcgttcgct ccaagctggg ctgtgtgcac gaacccccccg
5341 ttcagcccga ccgctgcgcc ttatccggta actatcgtct tgagtccaac ccggtaagac
5401 acgacttatc gccactggca gcagccactg gtaacaggat tagcagagcg aggtatgtag
5461 gcggtgctac agagttcttg aagtggtggc ctaactacgg ctacactaga aggacagtat
5521 ttggtatctg cgctctgctg aagccagtta ccttcggaaa aagagttggt agctcttgat
5581 ccggcaaaca aaccaccgct ggtagcggtg gtttttttgt ttgcaagcag cagattacgc
5641 gcagaaaaaa aggatctcaa gaagatcctt tgatcttttc tacggggtct gacgctcagt
5701 ggaacgaaaa ctcacgttaa gggattttgg tcatgagatt atcaaaaagg atcttcacct
5761 agatcctttt aaattaaaaa tgaagtttta aatcaatcta aagtatatat gagtaaactt
5821 ggtctgacag ttaccaatgc ttaatcagtg aggcacctat ctcagcgatc tgtctatttc
5881 gttcatccat agttgcctga ctccccgtcg tgtagataac tacgatacgg gagggcttac
5941 catctggccc cagtgctgca atgataccgc gagacccacg ctcaccggct ccagatttat
6001 cagcaataaa ccagccagcc ggaagggccg agcgcagaag tggtcctgca actttatccg
6061 cctccatcca gtctattaat tgttgccggg aagctagagt aagtagttcg ccagttaata
6121 gtttgcgcaa cgttgttgcc attgctacag gcatcgtggt gtcacgctcg tcgtttggta

FIG. 31C

6181 tggcttcatt cagctccggt tcccaacgat caaggcgagt tacatgatcc cccatgttgt
6241 gcaaaaaagc ggttagctcc ttcggtcctc cgatcgttgt cagaagtaag ttggccgcag
6301 tgttatcact catggttatg gcagcactgc ataattctct tactgtcatg ccatccgtaa
6361 gatgcttttc tgtgactggt gagtactcaa ccaagtcatt ctgagaatag tgtatgcggc
6421 gaccgagttg ctcttgcccg gcgtcaatac gggataatac cgcgccacat agcagaactt
6481 taaaagtgct catcattgga aaacgttctt cggggcgaaa actctcaagg atcttaccgc
6541 tgttgagatc cagttcgatg taacccactc gtgcacccaa ctgatcttca gcatctttta
6601 ctttcaccag cgtttctggg tgagcaaaaa caggaaggca aaatgccgca aaaaagggaa
6661 taagggcgac acggaaatgt tgaatactca tactcttcct ttttcaatat tattgaagca
6721 tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag aaaaataaac
6781 aaataggggt tccgcgcaca tttccccgaa aagtgccacc tgacgtc

FIG. 32A

The sequence of the CMV expression vector expressing human codon optimized Vargula luciferase under control of the CMV promoter (not the vargula luciferase sequence) is in bold.

CMV promoter bases: 209-863
Vargula luciferase gene: 907–
T7 promoter bases: 864-882
Polylinker bases: 889-907

gacggatcgggagatctcccgatcccctatggtcgactctcagtacaatc tgctctgatgccgcatagttaagccagtatctgctccctgcttgtgtgtt
ggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaaggcaag gcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcg
ctgcttcgcgatgtacgggccagatatacgcgttgacattgattattgac tagttattaatagtaatcaattacggggtcattagttcatagcccatata
tggagttccgcgttacataacttacggtaaatggcccgcctggctgaccg cccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagt
aacgccaatagggactttccattgacgtcaatgggtggactatttacggt aaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccc
cctattgacgtcaatgacggtaaatggcccgcctggcattatgcccagta catgaccttatgggactttcctacttggcagtacatctacgtattagtca
tcgctattaccatggtgatgcggttttggcagtacatcaatgggcgtgga tagcggtttgactcacggggatttccaagtctccaccccattgacgtcaa
tgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgta acaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggag
gtctatataagcagagctctctggctaactagagaacccactgcttactg gcttatcgaaattaatacgactcactatagggagacccaagcttggtacc
gagctc **ATGAAGATAATTATCCTTTCTGTGATTCTGGCTTACTGTGTTAC**
AGTGAATTGTCAGGATGCATGTCCAGTAGAGGCGGAACCGCCATCTTCTA
CCCCGACCGTACCAACCTCCTGCGAAGCTAAAGAAGGGGAGTGCATCGAT
ACAAGGTGCGCTACCTGCAAACGGGATATCCTGTCCGACGGACTTTGCGA
AAATAAACCCGGGAAGACCTGCTGTCGAATGTGTCAGTATGTCATCGAAT
GCCGGGTCGAGGCCGCCGGTTATTTTAGAACATTTTACGGTAAACGGTTT
AATTTCCAGGAACCCGGCAAATACGTACTGGCTCGCGGCACCAAGGGTGG
CGACTGGAGCGTCACCCTGACAATGGAAAACCTGGACGGGCAGAAAGGAG
CCGTGCTTACTAAAACTACCCTGGAGGTGGCGGGAGACGTAATTGACATC
ACTCAGGCAACGGCTGACCCAATAACCGTGAACGGAGGAGCTGATCCCGT
GATTGCAAACCCTTTCACTATTGGCGAGGTCACGATTGCCGTCGTCGAAA
TTCCAGGCTTCAACATCACAGTGATCGAGTTCTTCAAGCTGATCGTCATT
GATATCCTCGGCGGACGGTCCGTTCGCATCGCACCTGACACAGCCAACAA
GGGCCTGATCTCTGGCATTTGTGGTAACTTGGAAATGAATGATGCTGATG
ACTTCACAACGGACGCCGACCAACTGGCCATTCAACCTAATATCAACAAA
GAGTTTGATGGATGTCCCTTTTACGGAAATCCTTCAGACATCGAATACTG
CAAAGGCCTCATGGAACCGTACCGGGCCGTTTGCAGAAATAACATCAACT
TCTACTATTATACTCTGAGCTGCGCATTTGCATACTGTATGGGCGGTGAG
GAGAGAGCCAAACATGTGCTTTTCGACTATGTGGAGACCTGCGCCGCCCC
GGAGACTCGCGGTACCTGCGTCCTGAGCGGCCATACCTTCTATGACACCT
TCGATAAGGCTAGGTACCAGTTCCAAGGGCCTTGCAAAGAGCTCCTGATG
GCCGCAGATTGTTACTGGAACACTTGGGACGTCAAAGTTTCCCATCGGGA
CGTAGAGAGCTACACGGAAGTTGAGAAGGTGACCATCAGGAAGCAGAGTA
CCGTCGTAGACCTGATCGTCGACGGCAAGCAGGTAAAGGTAGGAGGCGTG
GACGTTAGTATTCCGTATTCTTCTGAAAATACGAGCATCTACTGGCAGGA
TGGAGACATTCTGACAACCGCCATCCTTCCAGAAGCTCTGGTGGTGAAGT
TTAACTTCAAGCAGCTGCTGGTAGTGCACATTCGCGACCCATTCGACGGG
AAAACCTGTGGGATTTGCGGCAACTACAACCAGGACTCAACTGACGATTT
CTTTGACGCCGAAGGGGCTTGCGCTCTTACCCCAAATCCGCCTGGATGCA
CCGAAGAGCAAAAGCCTGAAGCGGAACGGCTGTGCAATTCACTGTTTGAT
TCTTCAATAGATGAGAAATGCAACGTGTGTTACAAACCTGACCGCATCGC
ACGCTGCATGTATGAGTATTGCCTGAGAGGTCAACAAGGGTTCTGCGATC
ACGCGTGGGAATTTAAGAAAGAATGCTACATAAAGCACGGGGATACATTG
GAGGTGCCGCCAGAATGCCAGTAGtctagaaataattcttactgtcatgccaagtaagatgcttttctgtgctgcaat
agcaggcatgctggggatgcggtgggctctatggcttctgaggcggaaag aaccagctggggctctaggggtatccccacgcgccctgtagcggcgcat
taagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgcc agcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccac
gttcgccggctttccccgtcaagctctaaatcggggcatccctttagggt tccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggt
gatggttcacgtagtgggccatcgccctgatagacggtttttcgccctt gacgttggagtccacgttctttaatagtggactcttgttccaaactggaa
caacactcaaccctatctcggtctattcttttgatttataagggatttttg gggatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaa
cgcgaattaattctgtggaatgtgtgtcagttagggtgtggaaagtcccc aggctccccaggcaggcagaagtatgcaaagcatgcatctcaattagtca
gcaaccaggtgtggaaagtccccaggctccccagcaggcagaagtatgca aagcatgcatctcaattagtcagcaaccatagtcccgcccctaactccgc
ccatcccgcccctaactccgcccagttccgcccattctccgccccatggc tgactaatttttttatttatgcagaggccgaggccgcctctgcctctga
gctattccagaagtagtgaggaggcttttttggaggcctaggcttttgca aaaagctcccgggagcttgtatatccatttcggatctgatcaagagaca
ggatgaggatcgtttcgcatgattgaacaagatggattgcacgcaggttc tccggccgcttgggtggagaggctattcggctatgactgggcacaacaga

FIG. 32B

caatcggctgctctgatgccgccgtgttccggctgtcagcgcaggggcgc ccggttcttttgtcaagaccgacctgtccggtgccctgaatgaactgca
ggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcg cagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattg
ggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccga gaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatc
cggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagca cgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaaga
gcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgca tgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccg
aatatcatggtggaaaatggccgcttttctggattcatcgactgtggccg gctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgata
ttgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttac ggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttga
cgagttcttctgagcgggactctggggttcgaaatgaccgaccaagcgac gcccaacctgccatcacgagatttcgattccaccgccgccttctatgaaa
ggttgggcttcggaatcgttttccgggacgccggctggatgatcctccag cgcggggatctcatgctggagttcttcgcccaccccaacttgtttattgc
agcttataatggttacaaataaagcaatagcatcacaaatttcacaaata aagcatttttttcactgcattctagttgtggtttgtccaaactcatcaat
gtatcttatcatgtctgtataccgtcgacctctagctagagcttggcgta atcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattc
cacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaa tgagtgagctaactcacattaattgcgttgcgctcactgcccgctttcca
gtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcgg ggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgac
tcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaa ggcggtaatacggttatccacagaatcaggggataacgcaggaaagaaca
tgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttg ctggcgtttttccataggctccgcccccctgacgagcatcacaaaaatcg
acgctcaagtcagaggtggcgaaacccgacaggactataaagataccagg cgtttccccctggaagctccctcgtgcgctctcctgttccgaccctgccg
cttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttc tcaatgctcacgctgtaggtatctcagttcggtgtaggtcgttcgctcca
agctgggctgtgtgcacgaacccccgttcagcccgaccgctgcgccttа tccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgcc
actggcagcagccactggtaacaggattagcagagcgaggtatgtaggcg gtgctacagagttcttgaagtggtggcctaactacggctacactagaagg
acagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaag agttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtt
ttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaa gatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactc
acgttaagggattttggtcatgagattatcaaaaaggatcttcacctaga tccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgag
taaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctc agcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgt
agataactacgatacgggagggcttaccatctggccccagtgctgcaatg ataccgcgagacccacgctcaccggctccagatttatcagcaataaacca
gccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcct ccatccagtctattaattgttgccgggaagctagagtaagtagttcgcca
gttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtc acgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaa
ggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttc ggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcat
ggttatggcagcactgcataattctcttactgtcatgccatccgtaagat gcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgt
atgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgc gccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcgg
ggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaa cccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgt
ttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataa gggcgacacggaaatgttgaatactcatactcttccttttcaatattat
tgaagcatttatcagggttattgtctcatgagcggatacatatttgaatg tatttagaaaaataaacaaatagggttccgcgcacatttccccgaaaag
tgccacctgacgtc

MULTIPLEX ASSAYS WITH MULTIPLE LUCIFERASES REPORTERS AND USES THEREOF

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/238,146, filed on Aug. 29, 2009, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention concerns the field of luciferase reporters useful in biological and biochemical assays.

BACKGROUND OF THE INVENTION

Luciferases are enzymes that catalyze reactions that emit light. Luciferases are named according to their source organisms such as beetles (firefly) or marine organisms. Examples of bioluminescent marine animals include: *Renilla*, also known as sea pansies, which belong to a class of coelenterates known as the anthozoans. In addition to *Renilla*, other representative bioluminescent genera of the class Anthozoa include *Cavarnularia, Ptilosarcus, Stylatula, Acanthoptilum*, and *Parazoanthus*. All of these organisms are bioluminescent and emit light as a result of the action of an enzyme (luciferase) on a substrate (luciferin) under appropriate biological conditions.

Different luciferases have different properties with regard to substrate specificity and intensity of light emission and stability of the bioluminescent signal, which is commonly measured by a luminometer. Luciferases are useful as transcriptional reporter genes and in imaging reporter gene expression in living subjects and many other applications in molecular biology.

Certain luciferases, such as those that utilize *cypridina* luciferin (vargulin) as a substrate, can be useful reporters because of their strong luminescent signal and the fact that they are secreted in the native form. However *cypridina* luciferin (vargulin) is very difficult to synthesize (usually involving an 18-step chemical synthesis). The limiting supply and the cost of the material have made the assay difficult to commercialize.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides modified luciferases, methods of making modified luciferases, and methods of using modified luciferases.

In one aspect, the present invention provides an isolated polynucleotide that encodes a modified *Luciola Italica* (also referred to as *L. Italica*) luciferase. In a further aspect, the modified *L. Italica* luciferase shows increased luciferase activity when expressed in mammalian cells as compared to a non human codon optimized mutant *L. Italica* luciferase.

In an embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that shows an approximately 1000-fold increased luciferase activity when expressed in mammalian cells as compared to a non human codon optimized mutant *L. Italica* luciferase.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is a red-emitting luciferase with an emission maximum of approximately 617 nm.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is human codon-optimized.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is a green-emitting luciferase with an emission maximum of approximately 550 nm.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that includes a secretory signal at its amino terminal end. In a still further embodiment, the secretory signal is a chymotrypsinogen secretory signal.

In one aspect, the present invention provides assays utilizing any of the modified luciferases discussed herein. In a further aspect, the assays are multiplexed reporter assays.

In one aspect, the present invention provides an isolated polynucleotide that encodes a modified *Renilla* luciferase. In a further aspect, the modified *Renilla* luciferase shows increased activity and stability over a native human codon optimized *Renilla* luciferase.

In an exemplary embodiment the present invention provides a modified *Renilla* luciferase that is a green-emitting *Renilla* luciferase.

In a further embodiment and in accordance with any of the above, the invention provides a modified *Renilla* luciferase that includes a secretory signal at its amino terminal end.

In one aspect, the present invention provides multiplexed luciferase assays comprising at least two different luciferase reports, where the at least two different luciferase reporters emit at two different wavelengths and/or utilize different substrates.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows data for relative luciferase stability for a *Cypridina* assay conducted using reagents without sodium chloride (VLAR-1) and with sodium chloride (VLAR-1 with sodium chloride).

FIG. **2** shows data for time course of activity in a *Cypridina* assay using 5 μl of sample (FIG. **2**A) or 20 μl of sample (FIG. **2**A).

FIG. **3**A-B shows the sequence of a green *Renilla* luciferase plasmid (SEQ ID NO: 1).

FIG. **4** shows the sequence of a modified red firefly luciferase with a secretory signal (SEQ ID NO: 2).

FIG. **5** shows data from a *Cypridina* luciferase assay in varying concentrations of sodium chloride.

FIG. **6** shows data from a *Renilla* luciferase assay with and without stabilizer (NP40).

FIG. **7** shows data comparing luciferase activity of native human codon optimized *Renilla* luciferase and a mutant *Renilla* luciferase of the invention.

FIG. **8** shows data comparing luciferase activity of human codon optimized and non-human codon optimized red-emitting *L. Italica* luciferase.

FIG. **9** shows data comparing luciferase activity of human codon optimized and non-human codon optimized green-emitting *L. Italica* luciferase.

FIG. **10** shows data comparing luciferase activity of intracellular red-emitting *L. Italica* luciferase and secreted red-emitting *L. Italica* luciferase.

FIG. **11** shows data comparing luciferase activity of secreted red *L. Italica* luciferase in the lysate and the supernatant from HEK293 cells.

FIG. **12** shows kinetics of luciferase activity in (A) Red *Luciola* luciferase, (B) *Guassia* luciferase, (C) *Cypridina* luciferase, and (D) Green *Renilla* luciferase.

FIG. **13** shows emission spectra from (A) a double reporter assay with *Vargula* and Red *Italica* luciferases and (B) a triple reporter assay with *Vargula*, Green *Renilla* and Red *Italica* Luciferases.

FIG. **14** shows kinetics data of a *Gaussia* luciferase assay using a GAR-1 reagent.

FIG. **15** shows data comparing stabilities of *Gaussia* luciferase assays using the GAR-2 reagent are in the presence of a stabilizer (FIG. **15**A) and in the absence of a stabilizer (FIG. **15**B).

FIG. **16** shows data related to relative luciferase activity of a firefly luciferase assay.

FIG. **17** shows data of relative luciferase activity of a *Cypridina* luciferase assay.

FIG. **18** shows data comparing luciferase activity of a modified *Vargula* luciferase of the invention in the lysate and the supernatant from mammalian cells.

FIG. **19** shows kinetic data for luciferase activity in a *Cypridina* luciferase assay.

FIG. **20** shows data comparing relative luciferase activity of Green *Renilla* luciferase in the absence (top panel) and presence (bottom) of a stabilizer.

FIG. **21** shows data from a firefly luciferase assay in the presence (square) and absence (diamonds) of a stabilizer.

FIG. **22** shows data from a dual assay of the invention utilizing firefly and *Cypridina* luciferases.

FIG. **23** shows data from a dual assay of the invention utilizing *Cypridina* (Panel A) and *Renilla* (Panel B) luciferases.

FIG. **24** shows emission spectra from a dual assay of the invention utilizing *Vargula* and Green *Renilla* luciferases.

FIG. **25** shows data from a triple assay of the invention utilizing *Cypridina*, firefly and *Gaussia* luciferases.

FIG. **26** shows emission spectra from a triple assay of the invention utilizing *Cypridina*, Green *Renilla* and Red *Italica* luciferases.

FIG. **27** shows the sequence of a red firefly luciferase of the invention (SEQ ID NO: 5).

FIG. **28** shows emission spectra from a dual assay of the invention utilizing *Vargula* and Red *Italica* luciferases.

FIG. **29** shows emission spectra emission spectra from a dual assay of the invention utilizing (A) *Gaussia* and Red *Italica* luciferases and (B) Green *Renilla* and Red *Italica* luciferases.

FIG. **30** shows the sequence of a red emitting firefly human codon optimized luciferase of the invention (SEQ ID NO: 3).

FIG. **31** shows the sequence of a human codon optimized green firefly luciferase of the invention (SEQ ID NO: 4).

FIG. **32** shows the sequence of a human codon optimized *Vargula* luciferase of the invention (SEQ ID NO: 6).

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing devices, formulations and methodologies which are described in the publication and which might be used in connection with the presently described invention.

Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polymerase" refers to one agent or mixtures of such agents, and reference to "the method" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention. It will be apparent to one of skill in the art that these additional features are also encompassed by the present invention.

Overview

The present invention provides modified luciferases and/or combinations of luciferases, and methods of utilizing those luciferases in reporter gene assays. In addition, the invention provides reagents that provide increased stability and activity in assays using luciferase reporters.

The present invention provides modified (also referred to herein as "mutant" or "variant") luciferases showing improved activity over wildtype luciferases or other modified luciferases known in the art reported to have improved properties for reporter gene assays or in vivo imaging applications. As used herein, "wildtype luciferases" refers to any luciferase that occurs in nature.

In certain aspects, the present invention provides modified luciferases that show brighter luminescence when expressed in mammalian cells as compared to the luminescence seen when wildtype luciferases are expressed in mammalian cells. The present invention also provides a method of expressing luciferase as a very bright intracellular reporter (not secreted) by sequence modification to increase its utility as an intracellular reporter in multiplexed assays and for imaging applications. The present invention also provides a composition for assays utilizing luciferases that lowers the cost and increases the efficiency and sensitivity of the assay by altering the reaction conditions such that high luminescence is produced using significantly less amount of luciferin.

The present invention further provides reagents for assays utilizing modified luciferases of the invention as well as mammalian expression vectors expressing secreted and intracellular luciferases.

In further embodiments the present invention provides sequence modifications (human codon optimization) to nucleotides encoding luciferases which result in an approximately 1000-fold increase in luciferase expression in transfected mammalian cells compared to the non-human codon optimized versions of these genes.

In further embodiments, the invention provides novel secreted reporter modified luciferases that are about 5 to about 35 fold brighter than wildtype luciferases. Such luciferases are used in accordance with the present invention as stand alone reporters or in multiplexed luciferase assays in combination with one or more other luciferases. As will be appreciated, combinations of luciferases for multiplexed assays of the invention can include both wildtype and modified luciferases.

In further aspects, the present invention provides assay compositions for measurement of modified luciferases of the invention as single luciferase assay formats. In further aspects of the invention, assay compositions are provided that enable simultaneous measurement of at least two different reporters in cell lysates or supernatants using a single assay solution. The luciferase activities of multiple reporters are analyzed by exploiting spectral differences in the emission maxima of the different luciferases.

Improved luciferases used in the present invention include without limitation: (i) a red-emitting firefly luciferase (Red-Fluc) from the Italian firefly *Luciola Italica* (emission max 609 nm), including intracellular (non-secreted) variants and secreted variants generated by fusing a chymotrypsinogen secretory signal sequence to the amino terminal end of the luciferase; (ii) a green-emitting firefly luciferase (Green-Fluc) from the Italian firefly *Luciola Italica* (emission max 550 nm), including intracellular (non-secreted) variants and secreted variants generated by fusing a chymotrypsinogen secretory signal sequence to the amino terminal end of the luciferase; (iii) a *Cypridina* Luciferase or *Vargula* luciferase (VLuc) from the marine ostracod *Vargula Hilgendorfi*, a secreted luciferase (emission max 395 nm or 462 nm depending on the substrate used); (iv) *Vargula* luciferase that has been modified at the C-terminal end with a KDEL sequence (endoplasmic reticulum retention signal) so that it is expressed intracellularly-VLuc-KDEL; (v) a modified secreted blue-emitting (emission max 480 nm) *Renilla* luciferase (B-Rluc) which is brighter and more stable than native *renilla reniformis* luciferase; (vi) a green emitting secreted *Renilla* luciferase (emission max 535 nm) modified to be secreted by fusing a synthetic secretory signal encoding gene sequence in frame with the gene encoding the green emitting modified of *renilla* luciferase; (vii) a *Gaussia* luciferase (emission max 482 nm) either native secreted (Gluc) or modified to be expressed intracellularly (Gluc-KDEL).

Luciferases of the Invention

Modified luciferases of the present invention show increased signal magnitude and stability. In certain embodiments, modified luciferases of the invention show at least a 1, 2, 3, 4, 5, 10, 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000-fold increase in the magnitude of the signal over signals seen with wildtype luciferases.

Modified luciferases of the invention may be intracellular (i.e., not secreted), or they may be modified to be secreted. In further embodiments, modified luciferases of the invention are engineered to further express a secretory signal, general at the amino terminal end. In some embodiments, the secretory signal is a synthetic sequence. In specific embodiments, the synthetic sequence is MLLK VVFA IGCI WQA (SEQ ID NO: 7). In yet further embodiments, the secretory signal is any signal that can induce secretion of the encoded protein, including without limitation an interleukin-2 secretory signal and a chymotrypsinogen secretory signal.

Vargula Luciferases of the Invention

In some aspects, the present invention provides a *Cypridina* Luciferase or *Vargula* luciferase (VLuc) from the marine ostracod *Vargula Hilgendorfi*, which is a secreted luciferase (emission max 395 nm or 462 nm depending on the substrate used).

In further aspects, the present invention provides a modified *Vargula* luciferase that shows increased signal and stability. In certain embodiments, the modified *Vargula* luciferase of the invention is human codon optimized to increase expression in mammalian systems. In further embodiments, a modified *Vargula* luciferase of the invention includes a wildtype or a native human codon optimized luciferase with the last two amino acids have been mutated CQ to SN (S=serine, N=asparagine). In still further embodiments, the present invention provides a mammalian vector expressing modified human codon optimized *Vargula* luciferase expressing intracellular *Vargula* luciferase. This sequence is the same as the wildtype or native human codon *Vargula* luciferase with the last two amino acids mutated CQ to SN (S=serine, N=asparagine) and with a KDEL (endoplasmic reticulum retention) sequence added after the C-terminal asparagine residue.

Firefly Luciferases of the Invention

In some aspects, the present invention provides a red-emitting firefly luciferase (Red-Fluc) from the Italian firefly *Luciola Italica* (emission max 609 nm) and a green-emitting firefly luciferase (Green-Fluc) from the Italian firefly *Luciola Italica* (emission max 550 nm

In further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases. Such human codon optimized red-emitting *L. Italica* luciferases show significantly increased activity over wildtype red-emitting *L. Italica* luciferases (see FIG. **8**). In still further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases according to the sequence provided in FIG. **30** (SEQ ID NO: 3). In still further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 3. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 3.

In still further embodiments, the present invention provides secreted red-*Italica* luciferases. FIG. **10** shows a comparison of luciferase activity of a human codon optimized red-emitting *L. Italica* luciferases fused to a chymotrypsinogen secretory signal to a non-secreted form of the human codon optimized red-emitting *L. Italica* luciferase. As discussed above, a number of different secretory signals can be used to produce secreted forms of modified luciferases of the invention. However, for red firefly luciferase, not all secretory signals produce a secreted luciferase. For example, popular signal sequences such as the N terminal 16 amino acid sequence of *Gaussia* luciferase and the Interleukin 2 secretory sequence do not successfully produce a secreted form of red emitting firefly luciferase.

Fusing a chymotrypsinogen secretory signal to a human codon optimized red-emitting *L. Italica* luciferases did successfully produce a secreted form of this luciferase. In some embodiments, the present invention provides a red firefly luciferase (also referred to herein as "red-emitting luciferase" and "red-emitting *L. Italica* luciferase") that is modified to include a synthetic secretory signal. In certain embodiments, the modified red firefly luciferase is encoded by the polynucleotide has the sequence provided in FIG. **4** (SEQ ID NO: 2). In still further embodiments, the present invention provides a luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 2. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 2. The underlined portion of FIG. **4** is the secretory signal. FIG. **11** shows a comparison of luciferase activities in supernatants and lysates of HEK293 cells transfected with a secreted red *Italica* Luciferase of the invention.

In further embodiments, the present invention provides human codon optimized sequences of green-emitting *L. Italica* luciferases. Such human codon optimized green-emitting *L. Italica* luciferases show significantly increased activity over a previously described thermostable mutant of green-emitting *L. Italica* luciferase (B. R. Branchini et al., Analytical Biochemistry, 361 (2): 253-262 (2007)—see FIG. **9**). In still further embodiments, the present invention provides human codon optimized sequences of green-emitting *L. Italica* luciferases according to the sequence provided in FIG. **31** (SEQ ID NO: 4). In still further embodiments, the present invention provides human codon optimized sequences of luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 4. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 4.

Renilla Luciferases of the Invention

In some aspects, the present invention provides a modified efficiently secreted blue-emitting (emission max 480 nm) *Renilla* luciferase (B-Rluc), which more stable than the wild-type *renilla reniformis* luciferase, and a green emitting secreted *Renilla* luciferase (emission max 535 nm) modified to be secreted by fusing a synthetic secretory signal encoding gene sequence in frame with the gene encoding the green emitting modified of *Renilla* luciferase. Mammalian cells transfected with the secreted green *Renilla* luciferase mutant described here show approximately 35-fold higher luciferase activity compared to mammalian cells transfected with the native (human codon optimized) *Renilla* luciferase (see FIG. **7**). Further the secreted green *Renilla* luciferase shows excellent stability of the bioluminescent signal (without compromising signal intensity) when assayed using the *Renilla* luciferase assay reagent described in this application (with the stabilizer included, see FIG. **7**), thus making it an ideal reporter for High throughput screening applications.

In certain embodiments, the present invention provides a green-emitting *Renilla* luciferase plasmid sequence with the sequence pictured in FIG. **3** (SEQ ID NO: 1).

Gaussia Luciferases of the Invention

In some aspects, the present invention provides a *Gaussia* luciferase (emission max 482 nm) that is either native secreted (Gluc) or modified to be expressed intracellularly (Gluc-KDEL). Such *Gaussia* luciferases can be used in single, double and triple reporter assays as discussed in further detail herein in combination with any of the other luciferases discussed herein or known in the art.

Luciferase Assays of the Invention

In certain aspects, the present invention provides compositions that improve stability and signal for assays utilizing wildtype and/or modified luciferases of the present invention.

In some embodiments, sodium chloride is added to improve the stability of luciferase assays of the invention. In such embodiments, a concentration of sodium chloride is utilized that improves the stability of the bioluminescent signal without affecting intensity. In further embodiments, sodium chloride concentrations in the range of about 0.05 M to about 1 M are used to improve stability of luciferase assays of the invention. In still further embodiments, sodium chloride concentrations of about 0.05 to about 0.5, 0.1 to about 0.4, about 0.2 to about 0.3, and about 0.05 to about 0.2M are used in luciferase assays of the invention. In specific embodiments, sodium chloride is added to improve the stability of assays utilizing wildtype and/or modified *Vargula* luciferases.

In further embodiments, certain luciferase substrates are added to luciferase assays to improve the stability of the bioluminescent signal. In such embodiments, the substrate added as a stabilizer may be an additional substrate that is not the substrate upon which the luciferase itself acts. For example, in assays utilizing *Cypridina* luciferase, coelenterazine is added to the assay to stabilize the assay stability. Coelenterazine is an oxidizable luciferin that is easily prone to oxidation but is not a substrate for the *Cypridina* luciferase. As will be appreciated, any luciferase assay described herein can be further modified by adding substrates for other luciferases as a stabilizer.

In some embodiments, the concentration of luciferase substrate is adjusted to improve the magnitude and/or stability of the signal. In further embodiments, low (under 1 μM) concentrations of substrate is used to improve luciferase signals. For example, for *Cypridina* luciferase assays, about 1 to about 25 nM Vargulin are used in assays of the invention. In further embodiments, about 1-100, 5-90, 10-80, 15-70, 20-60, 25-50, and 30-40 nM Vargulin are used in assays of the invention. In further exemplary embodiments, substrates for the luciferase assays described herein (including *Cypridina, Gaussia* and *L. Italica* luciferases) are added in concentrations of from about 1 nM to about 250 μM. In still further embodiments, substrates are added in concentration of about 10 nM-200 μM, 50 nM-150 μM, 100 nm-100 μM, 150 nm-50 μM, 200 nM-25 μM, 300 nM-10 μM, 500 nM-1 μM.

In some embodiments, *Gaussia* luciferases of the invention are used with optimized reagents to produce increased activity. Kinetics of the *Gaussia* luciferase assay using the GAR-1 reagent is shown in FIG. **14**. Measurement of the luciferase activity in supernatants of cells (transfected with *Gaussia* luciferase) using GAR-1 reagent from Targeting systems showed increased activity from *Renilla* luciferase assays from another vendor. The data in FIG. **14** is presented as an average of triplicate determinations measured on a Turner TD2020 luminometer. GAR-1 reagent has been described in detail in US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to assay reagents for the *Gaussia* luciferase assay.

In certain embodiments, *Gaussia* luciferase assays of the invention utilize reagents stabilized with stabilizing agents. In one non-limiting example, the stabilizing agents include NP40 (Sigma) and/or coelenterazine. In certain embodiments, about 5 to about 200 μM coelenterazine is used. In still further embodiments, about 10-150, 20-125, 30-100, 40-75, 50-60 μM coelenterazine is used. In yet further embodiments, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 μM coelenterazine is used. Stability of *Gaussia* luciferase assays using the GAR-2 reagent are shown in FIG. **15**. Using the GAR-2B version of the *Gaussia* luciferase assay reagent, the bioluminescent signal remains very stable (FIG. **15**A) In the absence of the stabilizer, the signal intensity is a little higher initially but decays faster than in the presence of the stabilizer (FIG. **15**B). Note that the data presented in FIG. **15**A and B is an average of triplicate determinations measured on a Turner TD2020 luminometer. The GAR-2 and GAR-2B reagents are stabilized versions of the GAR-1 reagent discussed in US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to reagents for *Gaussia* luciferase assays. The GAR-2 reagent includes the composition GAR-1 with an additional 30 μM coelenterazine. GAR-2B reagent includes the composition GAR-1 with and additional 75 μM coelenterazine. Without being limited by theory, it is possible that the higher (approximately 3-fold) signal

intensity seen with the GAR-2B reagent is due to the higher concentration of coelenterazine. FIG. **12**B shows the stability of the *Gaussia* luciferase with the GAR-2 reagent including a stabilizer.

In certain embodiments, stability of firefly luciferase assays is improved using FLAR-1 reagents (Targeting Systems). FIG. **16** shows the results from experiments using the FLAR-1 reagent from Targeting Systems. In the experiments shown in FIG. **16**, the FLAR-1 reagent was added to the supernatant cell culture media.

Dual and Triple Luciferase Assays

In some aspects, the present invention provides dual luciferase assays based on spectral resolution of two or more different luciferases. As will be appreciated, these assays can include different wildtype luciferases, different modified luciferases, or a mixture of a wildtype and a modified luciferase. Such assays rely on differences in the emission spectra of the reporters used. In further embodiments, reagents are modified to allow for more efficient multiplexing. For example, when *Gaussia* luciferases are multiplexed with firefly luciferases, EDTA is omitted from the reaction mixture to allow efficient reporter activity.

FIG. **13**A shows the emission spectra of a dual reporter assay utilizing a *Vargula* and Red *Italica* luciferase of the invention. The luciferases were expressed in samples of transfected cells. The luciferases used in the experiments pictured in FIG. **13**A represent a modified red emitting firefly luciferase of the invention that is human codon optimized and intracellular (non-secreted) and a *Cypridina* luciferase of the invention that is from *Cypridina hilgendorfi* modified to be human codon optimized and secreted.

FIG. **13**B shows the emission spectra of a triple reporter assay utilizing *Vargula*, Green *Renilla* and Red *Italica* luciferases. These emission spectra were in samples of transfected cell lysates. The *Vargula* and red-emitting firefly luciferases are those as described above for FIG. **13**A and the Green *Renilla* luciferase is an improved secreted Green luciferase mutant as described in further detail herein.

All patents and other references cited in the specification are indicative of the level of skill of those skilled in the art to which the invention pertains, and are incorporated by reference in their entireties, including any tables and figures, to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to obtain the ends and advantages mentioned, as well as those inherent therein. The methods, variances, and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Also, unless indicated to the contrary, where various numerical values or value range endpoints are provided for embodiments, additional embodiments are described by taking any 2 different values as the endpoints of a range or by taking two different range endpoints from specified ranges as the endpoints of an additional range. Such ranges are also within the scope of the described invention. Further, specification of a numerical range including values greater than one includes specific description of each integer value within that range.

Thus, additional embodiments are within the scope of the invention and within the following claims.

EXAMPLES

Example 1

Transfection of Mammalian Cells with Modified Luciferases

HEK-293 cells were grown in DMEM/10% FBS (fetal bovine serum) and transfected with plasmids expressing either the human codon-optimized or non-human codon optimized forms of the red emitting and green emitting firefly luciferases (from *Luciola Italica*) under control of the CMV promoter. Transfections were performed using the Targefect F-2 reagent (Targeting Systems) using the manufacturers protocols. Forty eight hours post transfection, the cells were lysed using the cell lysis reagent (CLR-1) from Targeting Systems, Santee. 20 μl aliquots of the cell lysate were mixed with 100 μl of the FLAR-1 (firefly luciferase assay reagent from Targeting Systems).

Example 2

Cypridina Luciferase Assays with Increased Stability

Compositions were developed for achieving optimal performance of *Cypridina* luciferase assay reagents. These assays had improved stability of the bioluminescent signal without affecting the overall activity of the enzyme.

Vargulin is generally unstable and easily oxidized, making long term storage of this substrate difficult. However, Vargulin stored in an acidic buffer (66 mM monobasic potassium phosphate, pH 6-6.5) and stored at −80° C. was very stable and did not lose activity even when stored for several months. In contrast, Vargulin dissolved in a neutral to basic phosphate buffer (e.g. 200 mM dibasic potassium phosphate (ph 8)) is very unstable and begins to lose activity rapidly within a few hours at room temperature. *Cypridina* luciferase activity was

optimal when 200 mM dibasic potassium phosphate was used as the reaction buffer instead of 66 mM monobasic sodium phosphate. Hence 200 mM dibasic potassium phosphate was used as the reaction buffer. Concentrations of be 3-6 nM Vargulin were found to be effective, and these concentrations are much lower than what is generally used in such assays (see for example Wu et al (2007) *Biotechniques,* 42(3):290-292).

The *Cypridina* luciferase assay showed increased stability when sodium chloride was included in the reaction. For example, FIG. **1** shows the relative luciferase stability (RLS) between VLAR-1 (no sodium chloride) and VLAR-2 (VLAR-1+sodium chloride). Sodium chloride clearly stabilized the RLS. For the experiments in FIG. **1**, 20 μl of sample was added with 40 μl of VLAR solution for the assay followed by 20 μl of Vargulin substrate.

FIG. **5** shows a further titration experiment indicating that sodium chloride concentrations of around 0.5M provide increased stability over control reagents with no sodium. Further concentrations that are of use in stabilizing such assays include from about 25 mM to about 750 mM sodium chloride. For experiments in FIG. **5**, 5 μl of the indicated concentrations of sodium chloride solutions were added to 35 μl of VLAR buffer (20 mM dibasic potassium phosphate, pH=8.0). The assay was carried out by mixing 20 μl of sample with 40 μl of VLAR buffer (with sodium chloride) and then adding 20 μl of *Cypridina* luciferin.

Further stability of the bioluminescent signal as well as improvement in overall luciferase activity was observed when coelenterazine, another oxidizable luciferin easily prone to oxidation (but not a substrate for *Cypridina* luciferase) m was included in the assay composition. A 15 minute pre-incubtion was found to result in increased stability of the bioluminescent signal using sample volumes between 5 and 20 μl (roughly 40% drop in 26 minutes using an assay volume of 20 μl and 15% drop in 26 minutes using an assay volume of 5 μl—see FIGS. **2**A and **2**B. A concentration of coelenterazine that worked well to stabilize the reagent was 15 μM. Concentrations in the range of about 10 μM to about 50 μM can also be used. The inclusion of coelenterazine in the composition decreased the background of the assay by more than 10-fold (background reading dropped form 153.6 to 12.4) and also resulted in a 15% increase in the intensity of the bioluminescent signal. Controls in which buffers with identical composition (i.e., inclusion of coelenterazine but omission of *Cypridina* luciferin) showed no activity. Coelenterazine is not a substrate for *Cypridina* luciferase and can be used to safely reduce the background and increase stability when *Cypridina* luciferase is assayed alone or in combination with other luciferases (such as firefly luciferase) which do not use coelenterazine as a substrate. For the experiments shown in FIG. **2**, 5 or 20 μl of the sample (media supernatant) was mixed with 40 μl of the VLAR buffer (200 mM dibasic potassium phosphate, 50 mM NaCl). The firefly and *Cypridina* luciferase assay reagents can be mixed into a single solution which can be used to efficiently measure both *Cypridina* luciferase and firefly luciferase activity by spectrally resolving the luciferases using appropriate filers. However, the DTT concentration in the firefly luciferase assay reagent can affect activity in such situations, because the activity of both luciferases is decreased due to interference of DTT (present in low concentration in the firefly assay reagent with the *Cypridina* luciferase assay (there is almost a 10-fold drop in *Cypridina* luciferase activity). However, since the signal intensity of the *Cypridina* luciferase assay is very robust, the signal is still acceptable and improvement in *Cypridina* luciferase activity is observed if the DTT concentration in the firefly luciferase assay reagent is dropped to 2.5 mM (a 3 fold drop in activity of *Cypridina* luciferase is still observed). Single solution based dual assays in which *Cypridina* luciferase is multiplexed with Green emitting *Renilla* luciferase work very well without loss of activity of either *Cypridina* or *renilla* luciferase when the two solutions are mixed.

Example 3

Renilla Luciferase Assays Utilizing Modified *Renilla* Luciferases and Stabilizing Reagents

The secreted modified green *Renilla* luciferase of the present invention showed significantly greater activity over wildtype *Renilla* luciferase—see FIG. **7**. For the experiments pictured in FIG. **7**, HEK 293 cells were transfected with expression vectors expressing either native *Renilla* luciferase or the secreted Green *Renilla* luciferase mutant. Cells were lysed 48 hrs post transfection and assayed for luciferase activity.

Assays with and without stability assay reagents for green *Renilla* luciferase were investigated. FIG. **6** shows that assays conducted with stabilizer showed greater stability than those without. The composition of the *Renilla* luciferase assay reagent (no stabilizer) was: 30 μM coelenterazine, 0.4×PBS (Ca, Mg free), 0.027% NP40. The composition of the *Renilla* luciferase assay reagent (with stabilizer) was: 30 μM coelenterazine, 0.4×PBS (Ca, Mg free), 0.227% NP40. Stabilizer is 2% NP40 (a non-ionic detergent).

Example 4

Kinetics of Different Luciferases

Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the luciferase assay reagents supplied with the LiveResponse assay kit. These data are shown in FIG. **12**A: Red *Luciola* (firefly), luciferase, FIG. **12**B *Gaussia Princeps* luciferase (this is FIG. **15**C), FIG. **12**C: *Cypridina* luciferase, and FIG. **12**D: Green *Renilla* luciferase. Data represents mean of triplicate determinations.

Example 5

Comparison of Expression Vectors Expressing Modified *Vargula* Luciferases

Transfection protocols were as follows: HEK-293 cells were grown in DMEM/10% FBS (fetal bovine serum) and transfected with plasmids expressing wither the human codon-optimized to non-human codon optimized forms of native VLuc, HC-VLuc, sequence 1) or modified HC-VLucs under control of the CMV promoter. Transfections were performed using the Targefect F-2 regent (Targeting Systems) using the manufacturers protocols.

The stability of the bioluminescent signal of *Cypridina* Luciferase assessed using supernatants of HEK293 cells transiently transfected with the pCMV VLuc expression vector is shown in FIG. **17**.

In FIG. **19**, the stability of the bioluminescent signal of *Cypridina* Luciferase was assessed using supernatants from HEK 293 cells transiently transfected with the pCMV-VLuc expression vector. Samples were assayed using the VLAR-2

(VLAR-1 reagent from Targeting Systems with sodium chloride) of the *Cypridina* luciferase assay reagent.

Human codon optimization of the gene sequence encoding the VLuc led to a 5-fold improvement of luciferase expression in HEK-293 transfected with expression vectors containing the human codon optimized versions of the *vargula* luciferase genes compared to the native sequences (i.e. Non-human codon optimized sequences). Addition of the KDEL sequence at the C-terminal end results in intracellular expression of VLuc.

Example 6

Construction of Blue-Emitting (Blue Shifted) and Green Emitting Mutants of Secreted *Renilla* Luciferase for Use as Secreted Reporters in Single or Multiplexed Luciferase Assays

A synthetic signal peptide was deduced by rational design: MLLK VVFA IGCI VVQA (SEQ ID NO: 7). The sequence of this signal peptide was based on rational design using signal sequences from the secretory signals known in the art, including those available at: http://www.unitargeting.com/Resources/Trends07.pdf

Blue-Sifted Secreted *Renilla* Luciferase Mutants

Secreted mutants were constructed containing signal peptide fused to amino terminal region of the human codon optimized *renilla reniformis* luciferase with the following additional mutations which enable i) efficient refolding after secretion to obtain an active form of the enzyme (Loma Linda paper, cysteine 124 was mutated to alanine) and additional mutations to cause a shift in the emission max of *Renilla* luciferase. MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; N53Q; V146M. Emission maxima=475 nm

Secreted BLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; N53Q; V146M and the following eight additional mutations A55T, S130A, K136R, A143M, M185V, M253L, S287L. The 8 additional mutations increase intensity of the bioluminescent signal (Emission Maxima 475 nm)

Red Shifted *Renilla* Luciferase Mutants:

Secreted RLuc Sequence 1: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A, D162E

Secreted RLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L F262W. Emission Maxima 535 nm

Secreted RLuc Sequence 3: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L. Emission Maxima 535 nm

Example 7

Tests for Developing Assays for *Vargula* Luciferase

In some embodiments, different buffer solutions are used to improve assays utilizing wildtype and/or modified luciferases of the invention. In certain embodiments, a 1:1 mixture of 0.1 M Tris HCl and 75 mM sodium phosphate is used as the assay buffer.

Several different parameters were tested to develop an assay for *vargula* luciferase:

Effects of using either an acidic buffer (e.g., potassium phosphate pH 5-6.8), Tris HCl pH 7.4, Tris phosphate buffer pH (8-8.5) as well as varying assay volumes were tested. In general the use of acidic conditions significantly reduced the intensity of the bioluminescent signal (typically 5-10 fold) while increasing the stability somewhat. Using Tris HCL ph 7.4, the activity as the assay buffer resulted in 5-10 fold brighter bioluminescence but the luminescent signal was highly unstable.

Use of a buffer mixture (1:1) of 50 mM Tris HCl, pH 7.4 and 100 mM dibasic sodium phosphate resulted in improved stability of the bioluminescent signal without compromising the intensity of the bioluminescent signal. An interesting finding was that inclusion of 0.2 M NaCl further increased stability of the bioluminescent signal. Lastly the amounts of Vargulin needed for optimal activity using this buffered condition are very low (1-10 nM range) making the assay extremely useful and economical.

Increasing the concentration of Vargulin further did not increase stability of the assay further.

Stock Vargulin substrate solutions stored in an acidic condition pH (5.5-6) were relatively stable over several months when stored at −80° C.

Other parameters tested: Other stabilizers such as DTT (dithiothreitol), detergents like NP-40 or EDTA were unable to increase the intensity of the luminescent signal or improve stability of the assay. EDTA decreased the VLuc activity by at least 5-fold.

Thus one aspect of the invention concerns the following composition and variations thereof: 20 μl of cell supernatant assays with 50 μl of Tris/phosphate buffer, pH 8, 0.2 M NaCl, 10 μl of 5-100 nM vargulin in 66 mM potassium phosphate (monobasic). In certain assays, the effective concentration of vargulin in the assay mix is as low as 20 nM which is approximately 50-fold lower than that reported in the literature (see for example Wu et al (2007) Biotechniques, 42(3):290-292)

Comparison of luciferase activity in cells transfected with *vargula* luciferase with luciferase activity in cells transfected with firefly luciferases from *Photinus pyralis* or *Luciola* Italic showed that *vargula* luciferase was a much more sensitive reporter (10-20 fold improvement in bioluminescent signal compared to firefly luciferase, assay done in HEK-293 cells, all expression vectors were expressed luciferase under control of the CMV promoter). An exemplary assay protocol included: 20 μl aliquots of Cell supernatants (media with 5% serum) were mixed with 100 μl of assay dilution buffer (50 μl of 50 mM TrisHCl, 100 mM dibasic sodium phosphate, pH 8) and 10 μl of vargulin in sodium phosphate buffer pH 6 (final concentration of vargulin in reaction mix 10-25 nM). The sample was mixed well and bioluminescent activity was recorded in a Turner TD2020 luminometer integrated over a 20 sec time interval.

Example 8

Activity in Cell Supernatant and Cell Lysates of Cell Transfected with Either a Plasmid Vector Expressing Secreted *Vargula* Luciferase or an Intracellular Form of *Vargula* Luciferase

In cells transfected with the secreted form of modified *vargula* luciferase, 80% of the activity was secreted into the cell supernatant and only 20% is cell-associated.

FIG. **18** shows intracellular and secreted *Cypridina* luciferase activity. Luciferase activity in cell supernatants and cell lysates of cells transfected with a plasmid vector expressing secreted *vargula* luciferase. As shown in FIG. **18** cells transfected with the secreted form of modified *vargula* luciferase, 80% of the activity is secreted into the cell supernatant and only 20% is cell-associated.

In cells transfected with *vargula* luciferase modified at the C-terminal end with a KDEL sequence, approximately 95% of the activity was intracellular and 5% is secreted.

Example 8

Development of a Dual Reporter System Based on Blue and Red Shifted Mutants of Secreted *Renilla* Luciferase

Secreted mutants: Secreted mutants were constructed containing signal peptide fused to amino terminal region of the human codon optimized *renilla reniformis* luciferase with the following additional mutations which enable i) efficient refolding after secretion to obtain an active form of the enzyme (Cysteine 124 was mutated to alanine) and additional mutations to cause a shift in the emission max of *renilla* luciferase: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; N53Q; V146M. Emission maxima=475 nm

Secreted RLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations. C124A; N53Q; V146M and the following eight additional mutations A55T, S130A, K136R, A143M, M185V, M253L, S287L. The 8 additional mutations increase intensity of the bioluminescent signal. Emission Maxima 475 nm

RED SHIFTED *RENILLA* LUCIFERASE MUTANTS: Secreted RLuc Sequence 1: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A, D162E

Secreted RLuc Sequence 3: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L F262W. Emission Maxima 535 nm

Secreted RLuc Sequence 4: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; and the following eight additional mutations. A123S/D154M/E155G/D162E/I163L/V185L. Emission Maxima 535 nm

A single solution dual luciferase assay based on secreted *renilla* luciferase blue emitting (emission max at 475 nm) and green emitting mutants (emission max at 535 nm).

The mutations in the above sequences lead to the efficient expression of secreted *renilla* luciferase in the transfected cells. The two luciferases can therefore be used in combination as a dual reporter system and the luciferase activity of each luciferase in the transfected cells can be resolved by using appropriate filters. The reagent compositions for *renilla* luciferase assay reagents are described Walia, US Pat Appl Publ 2008074485, entitled Enhancing a Luminescent Signal, which is incorporated herein by reference in its entirety and in particular for all teachings related to *Renilla* luciferase assay reagents.

Example 9

Development of a Triple Reporter System Based on Red and Green Emitting Firefly Luciferases and *Gaussia* Luciferase/*Renilla* Luciferase

Composition of the *Gaussia* luciferase assay reagent (GAR-1) has been described in detail in a US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to assay reagents for the *Gaussia* luciferase assay. An assay reagent useful for simultaneous measurement of all there reporters in a single solution was designed by omitting EDTA from the composition of the *Gaussia* luciferase assay reagent and then including all the ingredients necessary for assay of firefly luciferase in a single composition. The rationale behind this is that the EDTA interferes with the firefly luciferase assay (magnesium is an important co-factor for firefly luciferase and EDTA chelates magnesium). The ingredients required for Firefly luciferase assay included in the assay composition were as follows—ATP, DTT. Firefly luciferin, magnesium sulfate, magnesium bromide (helps increase brightness of luminescent signal) and phosphate buffer.

The composition of the single solution for a triple reporter assay for measuring *Gaussia* luciferase or *Renilla* luciferase in combination with red and green emitting firefly luciferase is as follows:

0.1×PBS. 5.4 ml of 5% NP40 diluted to 1000 ml and add the following:

To 800 ml of the above solution add the following:

Tricine 3.227 g (20 mM)

1M Magnesium sulfate 0.7H2O 2.51 ml (2.67 mM

Magnesium bromide 0.6 H2) (1.07 mM)—add 2.14 ml of 500 mM stock solution

25 mM OTT (3.86 g)

530 μM ATP (2.72 g)

CoA (0.18 g)—optional

Adjust with sodium phosphate to pH 7.8

Add 940 μM D-Luciferin (fee acid)—253.81 mg

CDTA-0.8289 g

940 μM D-luciferin (free acid)—253.81 mg

CDTA-0.8289 g

0.8M Tris (0.02 M EDTA)—43.53 ml

Add GAR reagent without EDTA to a total volume of 1 liter

Dilute 100× coelenterazine substrate with the above solution to 1× just before use. Use normal 3 mg/5 ml absolute alcohol acidified with 30 μl of 2N HCl)

NOTE: This assay reagent does not contain enough cell lysis reagents. Hence cells have to be first lysed using 1× Cell Lysis Buffer (compatible with use of all luciferases (prepared from 5× stock solution described below:

Dilute the 5× Cell lysis buffer described below with water to 1× concentration and add to washed cells and shake at 400 rpm for 20 mins to lyse cells.

Composition of 5× Cell Lysis Buffer:

For 1 liter of Buffer

5 ml NP 40 (undiluted)

25 ml Tris HCl ph 8

1.45 g NaCl

50 ml glycerol

Example 10

Development of a Single Solution Triple Luciferase Reporter Assay Based on Red and Green Emitting Firefly Luciferases and *Vargula* Luciferase

A *vargula* luciferase-based triple reporter system was prepared by first preparing the *vargula* luciferase assay reagent (VLAR-1) and mixing it in a 1:1 ratio with the firely luciferase assay reagent (FLAR-T) to give the triple assay reagent TVLAR-1.

Assay protocol: To 20 μl of cell lysate add 100 μl of the TVLAR-1 reagent and read in the Victor luminometer (Perkin Elmer) or Varian (Promega) using appropriate filters.

Preparation of VLAR-1 Reagent:

Composition of the *Vargula* Luciferase Assay reagent is described below

500 ML OF 0.1 M TRIS HCL PH 8

500 ML of dibasic sodium phosphate 200 mM

200 ml of 5 nM Vargulin in 66 mM potassium phosphate pH 5.5

pH of final solution is 8-8.5

Composition of the FLAR-T Reagent

SOLUTION A: 0.1×PBS. 5.4 ml of 5% NP40 diluted to 1000 ml and add the following:

To 800 ml of the above solution add the following:

Tricine 3.227 g (20 mM)

1M Magnesium sulfate. 7H2O 2.51 ml (2.67 mM)

Magnesium bromide (0.6 H2) (1.07 mM)—add 2.14 ml of 500 mM stock solution

5 mM DTT (in some embodiments, any range between 5 mM and 30 mM can be used, including 5, 10, 15, 20, 25, 26, 27, 28, 29, and 30 mM)

530 μM ATP (2.72 g)

CoA (0.18 g)—optionally omitted

Adjust with sodium phosphate to pH 7.8

Add 940 μM D-Luciferin (free acid)—253.81 mg

CDTA-0.8289 g

940 μM D-luciferin (free acid)—253.81 mg

CDTA-0.8289 g

941 μM D-luciferin (free acid)−253.81 mg

CDTA-0.8289 g

0.8M Tris (0.02 M EDTA)—43.53 ml

ADD SOLUTION A to a total volume of 1 liter

NOTE: This assay reagent does not contain enough cell lysis reagents for effective lysis. Hence cells should first be lysed, e.g., using 1× Cell Lys is Buffer (compatible with use of all luciferases (prepared from 1× stock solution described below: Dilute the 5× Cell lysis buffer described below with water to 1× concentration and add to washed cells and shake at 400 rpm for 20 mins to lyse cells.

Composition of 5× cell lysis buffer:

For 1 liter of Buffer

5 ml NP 40 (undiluted)

25 ml Tris HCl ph 8

1.45 o NaCl

50 ml glycerol

Composition of Firefly luciferase assay reagent (for use of firefly luciferase as a single reporter gene).

20 mM tricine (179.2 3.55 g)

MgCo3 1.07 mM 0.55 g

Magnesium sulfate 2.7 mM (277 ml)

0.1 mM EDTA

20 mM DTT (4.25 g)

530 μM ATP (3 g)

CoA (0.198 g)

Add disodium phosphate 25 g to ph 7.8

Add 793 ml water before pH

470 μM D Luciferin free acid 279.2 mg

5×CCLR 307 ml

Composition of 5×CCLR:

0.8 M Tris 0.02 M EDTA pH 8-156 ml

Glycerol 500 ml

Triton X100 50 ml

CDTA-7.5 m moles (2.7 g)

DTT 10 mM 1.542 g total vol 1 liter.

Example 11

Development of a Single Solution Triple Luciferase Reporter Assay Based on Red and Green Emitting Firefly Luciferases and *Vargula* Luciferase

Addition of stabilizer does not significantly affect (i.e, there is very little decrease in signal intensity) intensity of bioluminescent signal of *Renilla* luciferase in supernatants and lysates. FIG. **20** (top panel) shows a *Renilla* assay performed with 10 μl of *Renilla* Lysate and 20 μl of *Renilla* Supernatant. Assay went as follows: 20 or 10 μl of sample (Supernatant or Lysate), 50 μl of RLAR-1 reagent (Targeting Systems). FIG. **20** (bottom panel) shows *Renilla* Assay was performed using the same volumes of lysate and supernatant as in the experiments in the top panel. Assay protocol was as follows: 10 or 20 μl of lysate or supernatant depending on the assay, 50 μl of the RLAR-1 reagent and an additional 8 μl of RLAR stabilizer for an increased stability profile for a time course reading. The stabilizer lowered the initial RLU reading (decreased from approximately 9000 to approximately 7000 rlu) but showed a much higher level of stability when observed over 30 minutes to 1 hour (FIG. **12**C). The RLAR-1 reagent is useful for high throughput screening (HTS) applications in which a large number of samples need to be assayed. In the absence of the stabilizer, the signal intensity decays faster than in the presence of stabilizer (FIG. **21**). Note: Data presented is average of triplicate determinations measured on a Turner TD2020 luminometer. In FIG. **21**, a time course was taken using the standard protocol of 10 μl lysate, 50 μl of RLAR reagent without stabilizer indicating drop in *Renilla* luciferase activity.

FIG. **22** shows the stability of the bioluminescent signal of *Cypridina* luciferase and firefly luciferase using the DLAR-3 reagent. This reagent is useful for HTS applications involving both *Cypridina* luciferase and the red-emitting *Luciola* luciferase. Note: Data presented is average of triplicate determinations measured on a Turner TD2020 luminometer. The DLAR-3 reagent (Targeting Systems) is a dual assay reagent based on secreted *Cypridina* luciferase and a secreted or intracellular red-emitting firefly luciferase.

FIG. **28** shows emission spectra of *Cypridina* and Firefly luciferases in samples of transfected cells (lysates or supernatants). The emission spectra were recorded on a Fluorolog-3 spectrofluorometer (Horiba Scientific, Japan) using a liquid nitrogen cooled CCD. The luciferases were assayed by mixing 200 ul of the sample with the appropriate luciferase assay reagent to obtain spectral profiles. Emission max of *Cypridina* Luciferase is 463 nm; Red *Italica* 617 nm.

Example 12

Double and Triple Luciferase Reporter Assays Based on *Renilla* Luciferase, Firefly Luciferase and *Vargula* Luciferase

Kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the DLAR-5 system are shown in FIG. **23**. Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the DLAR-5 luciferase assay reagents. The decay of the *renilla* luciferase signal shown in Panel B above can be greatly minimized (ie the bioluminescent signal can be rendered much more stable by addition of a *Renilla* luciferase stabilizer to the DLAR-5 buffer.

FIG. **24** shows Emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activities of *Cypridina*, Green *Renilla* luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of *Vargula* luciferase is 463 nm; Green *Renilla* luciferase is 527 nm. Note that the data presented in this application is performed with the green-emitting mutant that emits at 527 to 530 nm (this is the variation in emission maxima seen and the luciferase is different in sequence, properties and emission maximum from the 535 nm emitting intracellular green emitting *Renilla* luciferase mutant described in US Patent Publication No.

20090136998, which is hereby incorporated by reference in its entirety and in particular for all teachings related to Green *Renilla* luciferase.

FIG. **25** shows kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the triple reporter system. Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were measured using the TLAR luciferase assay reagents (Targeting Systems).

FIG. **26** shows emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activities of *Cypridina, Renilla* and Red *Luciola Italia* luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of *Vargula* luciferase is 463 nm; Green *Renilla* luciferase is 527 nm and Red *Luciola Italia* luciferase is 617 nm.

The present invention also provides a single solution-based triple luciferase reporter assay involving *Cypridina* luciferase multiplexed with Green-emitting *Renilla* luciferase and Red-emitting Firefly luciferase. This assay is compatible with high throughput applications. This assay is also optionally in a format where the three luciferases can be assayed separately using three different assay reagents

SEQUENCE LISTING

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gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300

tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360

cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420

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tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660

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<210> SEQ ID NO 2
<211> LENGTH: 6839
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Modified red firefly luciferase with secretory signal

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<211> LENGTH: 6833

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A mammalian expression vector expressing Red emitting firefly luciferase (human codon optimized signal) under control of the CMV promoter)

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What is claimed is:

1. A multiplexed luciferase assay composition comprising:

multiple luciferase reporters, wherein at least two of the luciferase reporters are selected from the group consisting of a firefly luciferase, a *Renilla* luciferase, a *Gaussia* luciferase and a *Cypridina* (*Vargula*) luciferase, and

wherein the different luciferase reporters emit at different wavelengths and/or utilize different substrates, and

wherein the firefly luciferase is a red-emitting human codon optimized luciferase encoded by SEQ ID NO:3 with an emission maximum of approximately 617 nm; or

wherein the firefly luciferase is a green-emitting human codon optimized luciferase encoded by SEQ ID NO:4 with an emission maximum of approximately 550 nm; or

wherein the *Renilla* luciferase comprises A55T, S130A, K136R, A143M, M185V, M253L, and S287L mutations or AI23S, D154M, E155G, D162E, I163L, and V185L mutations compared to wildtype *Renilla* luciferase; or

wherein the *Vargula* luciferase is encoded by SEQ ID NO: 6; and

wherein at least one of the luciferase reporters comprises a secretory signal, wherein the secretory signal is peptide sequence of SEQ ID NO: 7.

* * * * *